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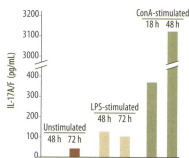
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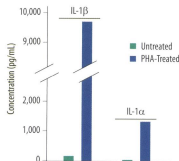
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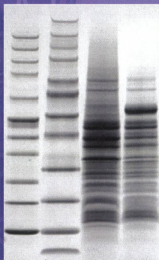
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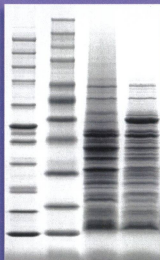
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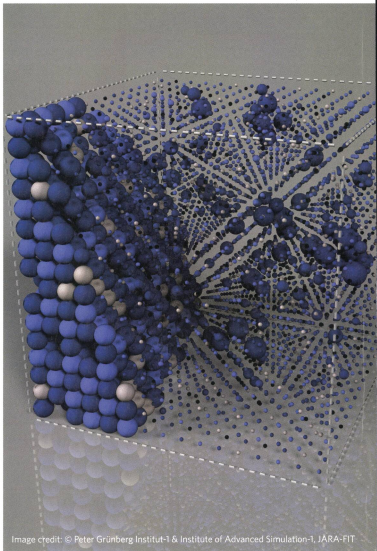


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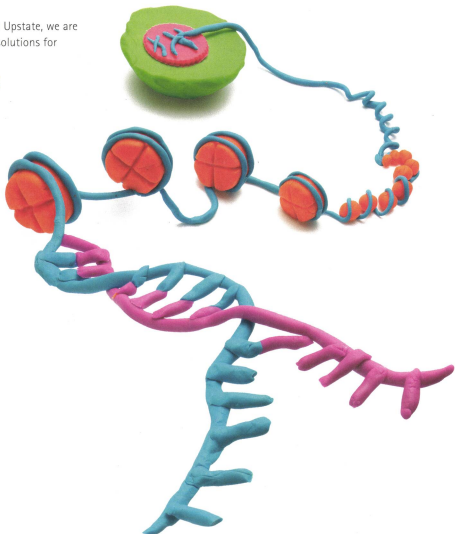
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Got Ethics?

We do. Ethics is doing the right thing, even if nobody is watching.

KAIMRC aims to disseminate biomedical ethic concepts into the health care system and community through education and public awareness. In an Islamic matrix of ethics and morality, we strive to set our own trends to formulate policies and guidelines, carry out and coordinate teaching and research, stimulate informed public debate, and provide a resource service for health professionals in our community and beyond. Ethical work behavior is a crucial part of success; all our employees possess a high degree of integrity and an outstanding level of professionalism.



At KAIMRC, a segment in the enormous healthcare system of King Abdulaziz Medical City, we felt the overwhelming sense of obligation towards both the healthcare profession and our institution to launch a Masters Degree program in Biomedical Ethics with the incorporation of Islamic Law. Healthcare professionals who show genuine interest in applying their knowledge and expertise to the ethical aspects of healthcare would be considered for program.

KAIMRC takes pride in its Bioethics Section, as it is home for the UNESCO chair of Saudi Arabia, held by its head Dr. Amin Kashmeery.

"Whoever works righteousness, man or woman, and has faith, verify, to them will We give a new Life, a life that is good and pure, and We will bestow on such their reward according to the best of their actions." - Holy Qur'an, Chapter 16, Verse 97

"It is better for a leader to make a mistake in forgiving than to make a mistake in punishing." - Prophet Mohammed (PBUH)

"In law a man is guilty when he violates the rights of others. In ethics he is guilty if he only thinks of doing so." - Immanuel Kant

"Ethics is knowing the difference between what you have a right to do and what is right to do." - Potter Stewart

Scientists Vacancies at King Abdullah International Medical Research Center

KAIMRC provides an opportunity to serve in a dynamic, multicultural environment with state-of-the-art facilities for competent and motivated persons who are willing to dedicate themselves and make a difference in the world of scientific medical research. If you are interested in being involved in demanding and innovative projects within one of the leading medical research centers worldwide, then search KAIMRC on Naturejobs.com to find our latest vacancies or apply with us by sending your resume to recruitment@kaimrc.med.sa. Please identify the position that you are applying to in the subject heading of your e mail.

Today's Question,
Tomorrow's Answer

KAIMRC Is Looking To Hire The Following:

1. Senior Research Scientist:

Education & Qualifications:

- Doctorate Degree (PhD) in Health Science or in a related discipline from an accredited university(Essential).

Experience:

- Minimum of twelve (12) years of experience in a well recognized research institution, coupled with two (2) years supervisory experience in a senior management position(Essential).

2. Research Scientist:

Education & Qualifications:

- PhD IN Science/Pathology or in a related discipline from an accredited University (Essential).
- Preferably post-doctoral research experience*

Experience:

- Eight (8) years working experience in clinical /research institution or Five (5) years experience with one or more publication as Principal Investigator in journal with 12 or more impact factor(Essential).

3. Associate research scientist:

Education & Qualifications:

- Doctorate Degree (PhD) in Health Science or equivalent from an accredited university (Essential).

Experience:

- Completion of Post Doctoral Fellowship (Essential).
- Minimum of three (3) years of experience in a clinical/research institution(Essential).

4. Research Assistant:

Education & Qualifications:

- Master's Degree in Health Science/ Health Management/Public Health or equivalent from an accredited college or university(Essential).

Experience:

- Minimum of four (4) years working experience in clinical research or scientific field(Essential).

5. Research Associate:

Education & Qualifications:

- Master's Degree in Health Science/ Health Management/Public Health or equivalent from an accredited college or university (Essential).

Experience:

- Minimum of seven (7) years of experience in a clinical/research institution(Essential).



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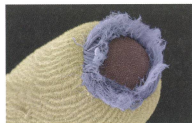
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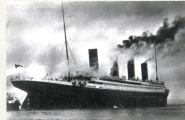
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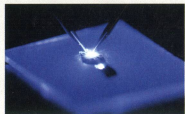
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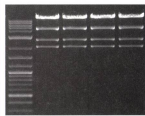
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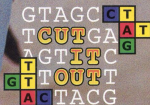
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THIS WEEK

EDITORIALS

WORLD VIEW The new model army needed to tackle financial crises **p.143**

ANTS Social insects take one for the diseased team **p.144**



MISSILE North Korea prepares controversial rocket launch **p.146**

Buyer beware

An investigation by Nature shows the scale of the market for unapproved stem-cell therapies in China. Hype and unrealistic hope must not be allowed to undermine genuine promise.

On the Internet, you can find advertisements for stem-cell remedies for every kind of disease or injury. Companies also promise that the cells will improve appearance or provide a 'rejuvenating' energy boost. The message — that stem-cell therapies need some work, but are an accepted part of medicine — is as clear as it is wrong.

But repeat this mantra enough — as it is repeated endlessly online — and the promises can start to seem real. In some places, they certainly look real. As we reveal on page 149, these 'cures' are offered in real clinics in China, where real nurses and doctors inject people with stem cells in various formulations from various sources — apparently convinced that they are helping patients. It looks and feels routine.

China has tried to crack down on unapproved treatments and it is not the only place where patients can buy these therapies: stem-cell companies also take advantage of gaps in regulation enforcement in the United States (see *Nature* **483**, 13–14; 2012). But in China the problem is more widespread.

Promoters of such unproven and unapproved 'treatments' liken stem-cell therapy to other once-revolutionary therapies, such as organ transplantation. Doctors confess that they can't guarantee that the stem cells will work, but they do guarantee that the procedures are safe. If they weren't, say advocates, we would hear about it. So why not try?

This circular logic makes the apparent infiltration of stem-cell technologies into the medical mainstream even more worrisome. The more willing patients and medics are to believe, the less they look for true clinical data, and the less doctors are forced to produce it.

Compare the emergence of stem-cell therapies with the introduction of psychosurgery. Like stem-cell practitioners today, doctors in the 1930s and 1940s felt that the need for lobotomy was urgent enough

to bypass the requirement for clinical evidence. Results were reviewed selectively, with pacifying brain damage sometimes taken as a stabilizing 'cure' for schizophrenia or nervous disorders. One promoter even shared the 1949 Nobel Prize in Physiology or Medicine for his part in developing the procedure. There was no long-term follow-up. In the end, doctors mutilated the brains of thousands of patients over the course of decades before critics were able to cast enough doubt on lobotomy to

"Acceptance is already overtaking clinical evidence, with no systematic follow-up."

halt its use. The widespread acceptance made it difficult for people to realize that these procedures were actually doing harm: you don't see a problem if you don't bother looking for it.

Of course, there is much legitimate research into stem cells, including many controlled clinical trials. It would be a shame if they were tainted by association with historical failures. However, judging from *Nature's* investigation in China, acceptance is already overtaking clinical evidence, with no attempt at systematic follow-up of treatments. If stem-cell therapies result in cancer or immunological disease, no one will know.

This does not stop people from outside China flocking to the country to take advantage of the stem-cell therapies offered there and promoted online with glowing endorsements. The clinics are certainly set up to make foreigners feel at home. Set aside from the teeming Chinese hospitals, stem-cell treatment centres have orderly nurse stations, well-lit rooms and good bedside care. What is lacking is controlled clinical trials, reliable data and government approval. If the dedicated medical workers at the clinics don't see the problems, they need to look harder. If they really want to help their patients, they should seek to prove that the treatments work, rather than just assuming that they do. ■

Honest work

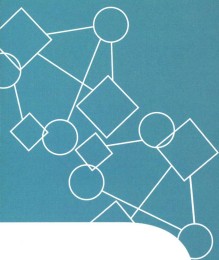
The plagiarism police deserve thanks for defending the honour of the PhD.

Last week, Hungary's President Pál Schmitt was forced to resign because of plagiarism detected in his 1992 PhD thesis on physical education. Tivadar Tulassay, rector of Budapest's prestigious Semmelweis University, showed admirable courage by standing up to the Hungarian establishment to revoke the thesis a few days earlier, after experts appointed by the university declared that Schmitt's thesis "failed to meet scientific and ethical standards". Tulassay, a cardiovascular researcher, has since assumed personal responsibility for his university's decision to revoke Schmitt's title.

The affair has remarkable parallels with that of Germany's former defence minister, Karl-Theodor zu Guttenberg, who resigned in March last year after his own PhD thesis, in law, had been revoked by the University of Bayreuth.

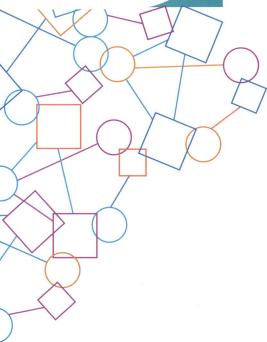
Like Schmitt, zu Guttenberg tried at first to deny plagiarism charges, then to underplay them, and he enjoyed powerful political support — until protests by a movement of honest PhD holders made his situation untenable. Plagiarism hunters have other prominent personalities in their sights, and are not necessarily going to be stopped just because a thesis is not in electronic form — if suspicion is high, they will digitize it themselves.

In many central European countries, an academic title is a decided advantage for a political career; clearly, some ambitious politicians think nothing of obtaining such a title by cheating. We can thank the plagiarism hunters — whatever their individual motives — for exposing dishonesty among those who govern us, and for defending the honour of a PhD. The only safe doctorate these days is an honestly acquired one. ■



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Scientists and bankers — a new model army

Bankers must now surrender more information on their activities. Scientists should use it to build better system-wide financial models, says John Liechty.

The financial system is in a credit-confidence trap. Like a badly balanced ship trying not to capsiz during a storm, banks and financiers are unwilling to make loans or accept collateral in exchange for securing debts — they fear being overwhelmed by the next wave of crisis. Even though the first sovereign default, in Greece, has passed, there is no obvious port in this storm.

How did the system get so far out of balance? And how can scientists — accustomed to modelling complex events to explain and make predictions — help to create a financial system that is more self-stabilizing? Existing financial models failed to predict the crisis of 2008 and the follow-on crisis of 2011–12. They missed the huge system-wide risks that developed as banks promoted an undisciplined supply of mortgages and created an increasingly complex web of relationships through legal contracts that transferred risk throughout the financial markets.

Financial bonds based on these mortgages (and other assets) were seen as risk-free and cheap, and banks used them as both capital and collateral. But when house prices plummeted, the bonds were useless for securing even short-term loans for the banks, which suddenly faced cash shortages. The banks held assets that were potentially worthless, and they were all interconnected. If one firm went down, everyone else was vulnerable.

Market forces function only if all risks are fairly priced. The system-wide risks that these bonds held were not taken into account, so the bonds were sold too cheaply. A clear scientific goal, therefore, is to build better system-wide models of the global financial system. Both the industry and regulators could use such models to judge financial risk and make decisions.

True, scientists are not blameless with regard to the recent collapse. They helped to create the models that the banks routinely used to measure risk. But those models lacked crucial data — on common holdings and trading behaviours, for instance, and on the interconnections between firms and the capacity of markets to execute trades.

For commercial reasons, banks have historically been reluctant to share this kind of information, but that is changing. Legislation in the United States now allows regulators to collect such data from banks, pension funds, insurance companies and other big players in the financial markets. Regulators in Europe are following suit, and hopefully Asia will as well. As a result, we will soon be able to model and identify potential system-wide risks.

To get an idea of the challenge of modelling system-wide financial risk, consider an indoor shopping mall that charges people to enter and exit. To model the movement of shoppers, we could build a purely statistical model of the door

traffic, and in most situations this would be sufficient. However, in extreme situations, such as a fire, the system would change dramatically. Shoppers would rush for the nearest exits and ticket-takers would get overwhelmed and close their doors. Then shoppers would rush to the next set of doors. In such cases, a statistical model would get it horribly wrong.

Equilibrium or structural models of the same system would track and predict the motives — and, therefore, the movement — of each individual. In normal times, these models are too complex and hard to calibrate — imagine trying to quantify all of the reasons that people go shopping. But in times of distress, as the shoppers' motives and behaviour converge (get out!), the model output improves.

To fully understand and predict the dynamics of a market in crisis, we have to understand the capacity of the market-makers (the ticket-takers at the door of the shopping mall) and the demand for assets when prices lurch significantly away from present levels (the number of shoppers trying to get out versus the number trying to get in). The new data will allow models to do this for the first time.

Clearly, regulators have a responsibility to build such models and to use them to monitor for potential crisis. To do this, they will need to leverage expertise among scientists by supporting and encouraging research in universities and labs, and by hosting the more applied work to maintain confidentiality. Banks should join this effort too, if only to avoid forcing regulators to use crude tools to set prices on these risks — through capital ratios or transaction taxes, for example.

Banks should work in parallel and form an industry group that collects system-wide data from its members, organizes resources for scientists to develop the necessary models, and creates a secure and confidential infrastructure for members to determine the price of system-wide risks. The industry already has a group that does something similar — the international Operational Riskdata eXchange Association, which shares operational-loss data among member firms.

Everyone would benefit if bankers were to engage with scientists to build the infrastructure needed to price system-wide risk. Banks could get feedback about common holdings and trading strategies, which would allow them to adjust their behaviour and avoid following the herd. Regulators would have extra market information to help them to determine when to act to ensure stability. And the rest of us could have increased confidence in the financial system. ■

**A CLEAR SCIENTIFIC
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BETTER
MODELS
OF THE GLOBAL
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RESEARCH HIGHLIGHTS

Selections from the
scientific literature

ECOLOGY

A look at backyard biodiversity

The choices city dwellers make when deciding which plants to cultivate in their gardens or yards could affect the function and health of the wider plant ecosystem.

Sonja Knapp at the Helmholtz Centre for Environmental Research in Halle, Germany, and her group compared the characteristics and diversity of plants in 137 residential yards with those of a nearby nature reserve. They found that yard plant species were more closely related to each other, shorter-lived, faster-growing and more likely to be self-pollinating.

As yard plants spread into natural habitats, the ability of those ecosystems to respond to environmental change could be reduced, the authors say.

Ecology <http://dx.doi.org/10.1890/11-0392.1> (2012)

ANIMAL BEHAVIOUR

Licking ants fight fungal infection

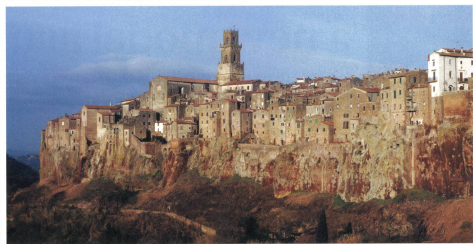
Healthy ants that rub up against infected counterparts or even lick pathogenic fungal spores off them may be immunizing themselves and, ultimately, protecting their whole colony.

Sylvia Cremer at the Institute of Science and Technology Austria in Klosterneuburg and her colleagues infected ants

(*Lasius neglectus*; pictured) with fluorescently labelled fungal spores (*Metarhizium antisopliae*) and released them among healthy members of their colony. The authors found that spores frequently transferred to healthy ants, resulting in low-level infection. Genetic analysis revealed that these minor infections upregulated a set of

immune-system genes that bolstered the ants' anti-fungal defences. Computer modelling suggests that this 'social immunization' actively stimulates the ants' immune systems, allowing the colony as a whole to fight infection.

PLoS Biol. 10, e1001300 (2012)



MARIA SUPERSTOCK

GEOLOGY

Hot tuff not so tough

Tuffs are volcanic rocks commonly used as building materials despite their notorious weakness — and at least one popular tuff could pose an even greater hazard in the event of a fire.

Michael Heap at the University of Strasbourg in France and his colleagues examined three types of tuff commonly used in buildings in the Neapolitan region of Italy (pictured). Two exhibited no reduction in strength after thermal stressing, but the most commonly used one,

known as Neapolitan yellow tuff, lost 80% of its compressive strength as temperatures reached 1,000 °C. This is explained by the fact that Neapolitan yellow tuff contains zeolite minerals that are sensitive to heat.

The team suggests that the results be considered in establishing regional fire codes and recommends similar tests for building tuff in other regions.

Geology 40, 311–314 (2012)

MOLECULAR BIOLOGY

MicroRNAs boost gene variation

Small RNA molecules that regulate and stabilize the expression of certain genes in humans may also promote and preserve variations in gene expression between individuals and ethnic groups.

Jian Lu and Andrew Clark at Cornell University in Ithaca, New York, examined the expression profiles of protein-coding genes that are influenced by microRNAs (miRNAs) and were obtained from multiple human populations. The authors compared these profiles with

those of genes not targeted by miRNAs. Expression of some of the miRNA-regulated genes varied little across populations, or between humans, chimpanzees and macaques. However, most differed from one individual to another and between ethnic groups.

Genome Res. <http://dx.doi.org/10.1101/gr.132514.111> (2012)

NANOTECHNOLOGY

Lasers sort particles by size

Gold nanoparticles have a range of biomedical uses, from detecting tumours to delivering drugs. However, their size is important because



it affects their optical and mechanical properties, as well as their toxicity. Martin Ploschner and his colleagues at the University of St Andrews, UK, report an efficient way to sort gold nanoparticles by size using laser light.

The team aimed green and red lasers at a thin layer of water containing a mixture of gold nanoparticles 100 and 130 nanometres in diameter. The green light's frequency matched that of the electrons in the smaller nanoparticles. This resonance enhanced forces acting on the particles, pushing them in one direction. The red light interacted with the larger particles, moving them in the opposite direction.

The researchers suggest that the method could sort nanoparticles more finely than current methods, which rely on centrifugation.

Nano Lett. <http://dx.doi.org/10.1021/nl204378r> (2012)

GENETICS

Fewer imprinted genes at re-count

Most mammalian cells have one maternal and one paternal copy of most genes, but some genes carry a molecular signature or 'imprint' that silences one copy. Tomas Babak at Stanford University in California and his team mapped the imprinted genes in mouse brains and found far fewer than recent estimates had suggested.

In 2010, two studies found more than 1,300 imprinted genes in the mouse brain, ten times more than traditional counts. The increase was attributed to improved RNA-sequencing technology. When Babak *et al.* repeated the experiments, they found only 13% of the imprinted genes first identified by the 2010 studies and uncovered statistical weaknesses that resulted in many false-positive signals. Using a different analytical approach, the authors identified roughly 50 new candidate imprinted genes.

Having a catalogue of imprinted genes is important for understanding why imprinting occurs and how it can go awry.

PLoS Genet. 8, e1002600 (2012)

MOLECULAR IMAGING

Follow the lymph vessels

Lymph vessels grow as wounds heal and cancers spread — a process that can be imaged in living animals, researchers demonstrate in mice.

Lymph vessels often sprout at sites of inflammation, and their growth has been linked to tumour metastasis. Sagrario Ortega at the Spanish National Cancer Research Centre in Madrid and her colleagues genetically engineered a mouse to express a luminescent protein under the control of the gene *Vegfr3*, a lymphatic marker.

The team imaged live mice, tracking vessel growth during embryo development, wound healing and inflammation. They also watched as lymph vessels grew at the edge of melanoma tumours and in lymph nodes infiltrated by the cancer. This vessel growth may aid the spread of cancer to distant organs, the authors say.

Proc. Natl Acad. Sci. USA

<http://dx.doi.org/10.1073/pnas.1115542109> (2012)

MATERIALS

A graphene window on liquids

By using graphene membranes as viewing 'windows', researchers have filmed nanocrystals growing in liquids at atomic resolution.

Studying structures in liquids at the atomic level is challenging because the imaging technique of choice, transmission electron microscopy, requires that samples be in a vacuum to maximize their interactions with the electron beam. Air-tight capsules can be used to enclose liquids, but are thick and made of materials

COMMUNITY CHOICE

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CANCER

Small, cancer-resistant mice

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Boosting the levels of a tumour-suppressor protein in mice makes them smaller and more metabolically efficient, as well as resistant to cancer.

Pier Paolo Pandolfi at Beth Israel Deaconess Medical Center in Boston, Massachusetts, and his colleagues genetically engineered mice to have additional copies of *Pten*, a gene that is mutated or deleted in many cancers. The mice are smaller than normal because they have fewer cells. When injected with a carcinogen, the animals developed tumours later than controls.

The transgenic mice burn energy at a higher rate. Cells from these mice consume less glucose than normal mouse cells but generate more ATP — the energy molecule created during cellular respiration — indicating a more efficient metabolism.

Increasing levels of the PTEN protein could offer a therapeutic approach to preventing both cancer and obesity.

Cell 149, 49–62 (2012)

that interfere with passing electrons, resulting in a blurred picture. Membranes made of graphene — atomically thin sheets of carbon atoms — are both impermeable to liquids and much more transparent to electrons.

Paul Alivisatos at the Lawrence Berkeley National Laboratory in Berkeley, California, and his colleagues used these graphene windows to create atomic-resolution movies of platinum nanocrystals clumping together in a liquid.

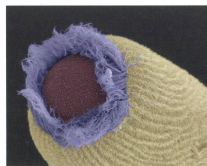
Science 336, 61–64 (2012)

ZOOLOGY

Vision with no nervous system

Sponge larvae can detect light despite lacking a nervous system or the photosensitive 'opsin' proteins found in all other known animal eyes. Instead, another pigment called cryptochrome may underlie the light-sensing ability of the sponge *Amphimedon queenslandica* (pictured), report Todd Oakley at the University of California, Santa Barbara, and his colleagues.

Cryptochromes mainly



absorb blue light and, in other animals, have been implicated in functions from setting circadian rhythms to sensing magnetic fields. The authors identified two cryptochrome genes in the sponge. One, *Aq-Cry2*, is expressed in the 'ring eyes' of *A. queenslandica* larvae and has an absorbance peak similar to the wavelengths that trigger larval activity.

Because eye evolution in other animals has always involved opsins, the use of cryptochrome represents a separate lineage of eye evolution, the team suggests.

J. Exp. Biol. 215, 1278–1286 (2012)

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SEVEN DAYS

The news in brief

POLICY

North Korea launch

North Korea has continued its plans to launch a long-range rocket in defiance of international pressure not to break a ban on missile testing. With a launch planned for between 12 and 16 April, the rocket (which North Korea says carries a weather satellite) was moved into position at the Sohae Satellite Launching Station on the country's northwestern coast last week. The plans have brought a swift end to February's agreement with the United States for food aid in return for a moratorium on nuclear testing, uranium enrichment and long-range ballistic missile development (see *Nature* 483, 128; 2012).

Astrophysics review

NASA's exoplanet-hunting space telescope, Kepler, is among nine astrophysics missions to have their lifetimes extended after a performance review by external scientists. Both Kepler and Swift, which detects γ -ray bursts, will run through to 2016. Although it recommended extensions for all the missions in its 3 April report, the review committee criticized the Hubble Space Telescope for a lack of transparency in reporting operating costs, and ordered Fermi, a γ -ray telescope, to cut costs by 10% each year from 2014. Only Spitzer, an infrared telescope, will be phased out earlier than its mission leaders wanted, in 2015. See go.nature.com/r6jgjo for more.

Carbon capture

The United Kingdom has relaunched a £1-billion (US\$1.6-billion) competition that will offer funding to companies that build commercial-scale facilities to capture and store carbon dioxide emissions from power



FRANÇOIS NOSTEN

Malaria drug resistance spreading

Malaria parasites that are resistant to the most effective current treatment — drugs based on artemisinin — are spreading in southeast Asia. Resistance was first confirmed in western Cambodia, close to Thailand, in 2008. But it is also emerging 800 kilometres westward, along the border of Thailand and Myanmar — where

villagers are shown being screened for malaria. Resistance will reach rates reported in western Cambodia within 2–6 years, says a 5 April study on the effectiveness of artemisinin treatments in more than 3,200 patients during 2001–10 (A. P. Phyto *et al.* *Lancet* <http://doi.org/hswh>; 2012). See go.nature.com/wefjya for more.

plants, to be in operation by 2016–20. The contest was first announced in 2007, but the bidders all pulled out. The relaunch, announced on 3 April, includes contracts guaranteeing the sale of the plants' electricity, which removes some financial risk. The competition also includes a further £125 million for research. See page 151 for more on carbon-capture plans around the world.

US public health

A committee convened by the US Institute of Medicine has recommended a suite of actions to remedy what it calls "dysfunction" in the funding, organization and capability

of the US public health-care system. Apart from urging a doubling of federal health-care spending, to be funded by taxing medical transactions, the 10 April report also endorsed more research into the effectiveness and value of public-health strategies. The 2010 Patient Protection and Affordable Care Act authorized a programme of research focused on similar issues — but that legislation is currently being challenged in the courts.

BUSINESS

Solar insolvencies

German solar-cell manufacturer Q-cells — which was the world's largest maker

of solar panels just five years ago — has filed for insolvency. The company, based in Bitterfeld-Wolfen, lost €846 million (US\$1.1 billion) last year and had been trying to restructure its debts, but gave up on 3 April. Three other German solar firms have filed for insolvency since December, as state subsidies decline and the market is flooded with low-cost photovoltaic modules and polysilicon raw material, much of it from China.

Imaging Alzheimer's

The US Food and Drug Administration on 6 April approved the first diagnostic test for plaques in the brain,

which are often associated with Alzheimer's disease. The test, developed by Eli Lilly, a pharmaceutical firm based in Indianapolis, Indiana, relies on a compound called florbetapir (Amyvid), which binds to amyloid plaques and can be imaged in living patients (see *Nature* 469, 458; 2011). Florbetapir assays will complement existing behavioural diagnoses of dementia, but researchers hope that the tests will eventually become a tool for early diagnosis and treatment of the disease.

RESEARCH

GM pigs on hold

A genetically modified pig intended for human consumption seems unlikely to reach the dinner table any time soon, after local hog producers decided to pull their funding for the project. The Enviropig, developed at the University of Guelph in Canada, contains a transgene that enables it to better absorb phosphorus from its food, in turn reducing the phosphorus content of its manure and counteracting problems such as algal blooms in waterways fertilized by phosphorus run-off. It has not yet been approved for human consumption, and Ontario Pork, a Guelph-based group for hog producers that had

put around Can\$1.3 million (US\$1.3 million) into the project, said last week that it would not provide any further funding because the genetics had been proven; interest from industry would be required to restart the research.

PEOPLE

Anti-doping row

Complaining of restrictions to his freedom of speech, an anti-doping researcher has resigned from the Swiss panel that oversees biological passports — biochemical profiles of athletes that help to detect doping (see *Nature* 475, 283–285; 2011). Michael Ashenden, who heads the Science and Industry Against Blood Doping Research Consortium in Gold Coast, Australia, said that he quit the Athlete Passport Management Unit in Lausanne because it added a confidentiality clause asking experts not to make public comments for eight years after they left the panel. The unit has just taken over the biological-passport system from the International Cycling Union in Aigle, Switzerland.

Heart-institute head

The US National Institutes of Health (NIH) has named cardiologist Gary Gibbons as director of the National Heart, Lung, and Blood Institute



(NHLBI). Gibbons (pictured) is currently at the Morehouse School of Medicine in Atlanta, Georgia, where he studies the genomics of vascular disease in minority populations. The NHLBI is the third-largest NIH institute, with a budget of roughly US\$3 billion, and has been without a permanent director since late 2009. Gibbons, replacing acting director Susan Shurin, plans to take up the post this summer.

FUNDING

Telescope rivalry

The 24.5-metre Giant Magellan Telescope (GMT), to be built in Chile, will not compete with its rival Thirty Meter Telescope in a contest for backing by the US National Science Foundation (NSF). With a mere US\$1.25 million for the winner, the contest was more about prestige than money. The NSF cannot

COMING UP

16–20 APRIL

The origin and evolution of life is the subject of a biennial gathering for astrobiologists, this year held in Atlanta, Georgia. go.nature.com/5ulicy

16–21 APRIL

In Panama City, the newly formed Intergovernmental Platform on Biodiversity and Ecosystem Services lays out its priorities for monitoring global ecology. go.nature.com/o3zm54

provide significant cash for either ground-based telescope until at least 2020, although the winner might find it easier to attract international partners. Last week, the GMT's board of directors declared that the project could proceed on its own. See go.nature.com/rwc7df for more.

Swedish bioscience

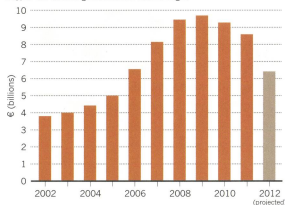
Sweden's government said on 3 April that the Science for Life Laboratory (SciLifeLab), an existing bioscience collaboration between four universities, will in 2013 expand into a national research institute for molecular biosciences and bioinformatics in Stockholm. It will eventually employ 1,000 scientists. The centre — which currently employs 300 scientists in Stockholm and Uppsala — is to receive 220 million Swedish kronor (US\$32.9 million) from the private Knut and Alice Wallenberg Foundation in Stockholm, and between \$25 million and \$50 million from pharmaceutical firm AstraZeneca. The government's contribution will be announced this autumn.

TREND WATCH

The Spanish government's austerity budget has lopped one-quarter off funding for research and development — much larger than the average 17% cut applied to other central-government departments. But public research funds may not be as badly hit as these figures suggest. More than half of overall funding relates to tax credits for technology transfer, for example, and last year almost half of those went unused. See go.nature.com/yqlqgl for more.

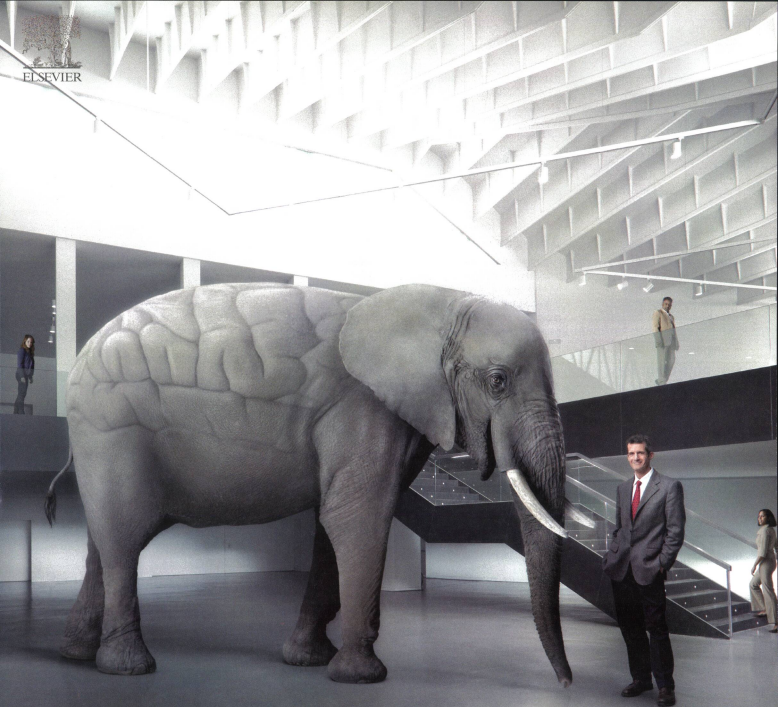
SPAIN SLASHES RESEARCH SPENDING

Spending on research and development in Spain drops 26% in the central government's draft budget.



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Prof. Francesco Visioli. Madrid Institute for Advanced Studies (IMDEA)-Food.

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NEWS IN FOCUS

VIROLOGY Parsing the lessons of the mutant-bird-flu controversy **p.150**

BIOTECHNOLOGY Roche is in hot pursuit of medical sequencing **p.152**

PLANETARY SCIENCE A wet blanket on dreams of a watery Mars **p.153**



EPIDEMIOLOGY Chronicling a generation, in flesh, blood and data **p.155**



It's boom time for firms selling stem-cell treatments, such as those derived from umbilical blood.

REGENERATIVE MEDICINE

China's stem-cell rules go unheeded

Health ministry's attempt at regulation has had little effect.

BY DAVID CYRANOSKI

Three months after the Chinese health ministry ramped up its efforts to enforce a ban on the clinical use of unapproved stem-cell treatments, a *Nature* investigation reveals that businesses around the country are still charging patients thousands of dollars for these unproven therapies.

The clinics operate openly, with websites promoting the treatments for serious disorders such as Parkinson's disease, diabetes and autism, and attract thousands of medical tourists from overseas. They advertise case studies of individual patients who they say have benefited from the treatments, and some have clinics in major hospital complexes, giving them

an air of mainstream acceptance. Stem-cell experts contacted by *Nature* insist that such therapies are not ready for the clinic and say that some may even endanger patients' health. But the Chinese government is struggling to enforce its ban.

In May 2009, the Chinese Ministry of Health classified stem-cell treatments as Category 3 medical technologies, defined as "high risk" and requiring the approval of a technical audit board before use. So far, no approvals have been granted. Despite this, "one 2009 estimate put the number of stem-cell companies based in China at around 100," says Doug Sipp, a stem-cell ethics and regulation researcher at the RIKEN Center for Developmental Biology in Kobe, Japan. In his view, "even after the

reform efforts by the Ministry of Health, the industry apparently continues to grow."

In January, recognizing the worsening situation, the health ministry announced a package of rules for the industry. Organizations using stem cells must register their research and clinical activities, the source of the stem cells and ethical procedures. The ministry asked local health authorities to halt any unapproved clinical use of stem cells in their regions. And it called for a nationwide moratorium on new clinical trials for stem-cell therapies, adding that patients in existing clinical trials should not be charged.

So far, however, the ministry's clampdown has proved ineffective. According to a Ministry of Health spokesman, not one clinic has registered in the required way, and *Nature* has found that many stem-cell clinics continue to offer treatments. Shanghai WA Optimum Health Care, for example, which has plush headquarters in a gated estate in one of the wealthiest areas of central Shanghai, claims success in using stem cells derived from umbilical cord or adipose tissue to treat a range of disorders, from autism to multiple sclerosis. Tony Lu, a member of the company's science and technology board, says that four to eight injections of such cells can treat Alzheimer's disease, at a cost of 30,000–50,000 renminbi (US\$4,750–7,900) per injection. According to the company's senior patient-liaison officer, Karina Grishina, autism can be treated with an adipose-tissue-derived cell injection for 200,000 renminbi, followed a few days later by a 50,000-renminbi injection of umbilical cord cells.

In Changchun, Tong Yuan Stem Cell claims to have treated more than 10,000 patients with a variety of disorders, including Parkinson's disease. A representative says that it also offers a one-year, four-injection autism treatment protocol using stem cells from aborted fetuses. Meanwhile, Beijing Puhua International Hospital's Stem Cell Treatment Center offers a four-to-five-injection protocol for autism, costing 205,000 renminbi.

Those clinics all claim success in treating patients, but none has published data from controlled clinical trials. Zhou Jingli, chief neurologist at Beijing Puhua, says that many of the company's autistic patients have shown marked improvements in their condition a couple of weeks after treatment. She agrees that controlled clinical trials are needed to ▶

► verify the efficacy of their treatments. "But," she asks, "who's going to pay?" She adds that first-hand experience with patients is enough to show that stem-cell treatment is worthwhile. "First, it's safe; second, it's effective. We know that," she says.

Leading stem-cell scientists think otherwise. Commenting on Tong Yuan's treatment for Parkinson's disease, Oliver Cooper, director of the Stem Cell Facility of the Neuroregeneration Institute at McLean Hospital in Belmont, Massachusetts, and a specialist in Parkinson's disease, says, "The products offered by Tong Yuan may provide anecdotal, poorly controlled, transient improvements in the patients, but Parkinson's-disease patients need long-term therapies."

"There are neither scientific nor clinical data to support the long-term benefits of haematopoietic- or neural-stem-cell therapies for Parkinson's patients," he adds. "In fact, it's not clear if the infused cells will survive for more than a few days in the patients."

Meanwhile, neurobiologist Ricardo Dolmetsch, an autism researcher at Stanford University in California, says, "The consensus in the autism research community, as well as in the stem-cell community, is that there is no scientifically valid reason for using stem cells

to treat autism spectrum disorders". He worries that, without the proper safety studies in place, the treatments could "lead to serious complications like cancer and autoimmune disease".

In addition to anecdotal case studies, some Chinese stem-cell companies bolster their reputations by claiming to have connections with leading politicians and scientists. A glossy 'information memorandum' from Shanghai WA Optimum Health Care contains pictures of staff with various local and central-government figures, including Li Keqiang, the powerful executive vice-premier of the State Council who is tipped to succeed Wen Jiabao as China's next premier.

It also lists Li Lingsong, director of the Peking University Stem Cell Research Center, as a member of its science and technology board. Li denies this. "I have so far nothing to do with WA," he told *Nature*, adding that he has asked the company to remove his name. WA also claims a strategic alliance with Harvard Medical School, although neither the medical school nor the Harvard Stem Cell Institute is aware of any such connection. Likewise, the University of California, Irvine, where WA claims to have research facilities, denies any formal relationship.

When pressed, all of the stem-cell clinics approached by *Nature* said that they were aware of the government regulations, and that they were necessary — but only for other clinics that were not operating safely. Most emphasized that their own businesses were entirely legitimate. *Nature* did find one company, Shanghai Puhua, which says that it has already stopped offering stem-cell treatments to comply with government regulations. And Beijing-based Wu Stem Cells will probably do likewise for the same reason, says company director Cheng Bo.

Bioethicist Zhai Xiaomei at Peking Union Medical College in Beijing, a member of one of the government's technical audit boards, was surprised to hear that any stem-cell companies were still operating. She says that the regulations are "absolutely clear" that companies must not administer unapproved stem-cell treatments.

A Ministry of Health representative told *Nature* that it was aware of the problem, and that it would be making greater efforts to clean up the stem-cell business.

Asked how WA operates despite the ministry's ban, Lu describes the regulations as "a grey area". Grishina agrees: "We're in China, so there are different stipulations." ■ **SEE EDITORIAL P.141**

BIO SAFETY

Post-mortem on mutant flu

Virus papers get green light but controversy highlights lack of global rules on biosafety.

BY DECLAN BUTLER

The dust is beginning to settle on the months-long controversy over two studies in which the H5N1 avian influenza virus was modified to be transmissible between mammals. But scientists and authorities still need to address the lack of international oversight for studies in which pathogens are deliberately made more dangerous, speakers emphasized at a two-day meeting held last week at the Royal Society in London. The meeting brought together scientists, research funders and experts in security, bioethics and foreign policy, just days after the US National Science Advisory Board for Biosecurity (NSABB) revised its earlier stance and recommended publication of the two studies.

In December 2011, the board recommended redaction of experimental details of the studies, on the basis of concerns about bioterrorism and the increased likelihood of accidental release of the viruses. But after considering revised versions of the manuscripts on 29–30 March, the NSABB voted unanimously



Yoshihiro Kawaoka can now publish his flu study.

in favour of full publication of the paper submitted to *Nature* by Yoshihiro Kawaoka of the University of Wisconsin–Madison, and 12–6 for publication of the content (although not the specific wording) of the paper submitted to *Science* by Ron Fouchier of the Erasmus

Medical Center in Rotterdam, the Netherlands.

Kawaoka presented his findings for the first time at the Royal Society meeting (see *Nature* <http://doi.org/hsn; 2012>), but Fouchier gave only a summary of his, saying that a more detailed description was prohibited by Dutch export-control laws, which require a permit to disseminate samples of, and information about, certain dangerous pathogens.

In December, the NSABB said that the information in the papers could help H5N1 surveillance efforts and so should be made available to experts on a need-to-know basis. A major factor in the board's change of heart was that subsequent international discussions concluded that there was no practical way to selectively share the data, and that national export controls may restrict distribution anyway, says Michael Imperiale, a virologist at the University of Michigan in Ann Arbor and a member of the NSABB.

This left the board with the stark choice of publishing either all or none of the research, with publication becoming "the only way for public-health authorities to know what to look

for", says Imperiale, who this time voted in favour of publishing both papers.

Some on the board were also influenced by clarifications to both papers — particularly those suggesting that Fouchier's viruses were not as pathogenic as they had initially seemed — and by presentations from Kawaoka and Fouchier indicating that some combinations of the mutations generated in their laboratories had already been seen in the wild. But Imperiale says that his vote was unaffected by the revisions. "I don't think the risks have changed; the authors have changed the host range and transmission properties of a deadly virus," he says. "I don't think the benefits have changed either."

Another factor in the NSABB's decision was the announcement on 29 March of a new US policy requiring that all publicly funded research on certain pathogens be assessed from the outset by the funding agency for the risk that it may be misused (see *Nature* <http://doi.org/10.1038/482012a>). Experts have generally welcomed the guidelines, which they say should, if properly applied, also help to avert future repeats of the H5N1 controversy, in which the NSABB learned of the papers only shortly before their planned publication.

Arthur Caplan, a bioethicist at the University of Pennsylvania in Philadelphia, was one of several speakers at the Royal Society meeting to argue that it would be a "mistake" to think that the issues raised by the papers are now fully resolved. Besides bioterrorism, a major concern is that publishing them will result in worldwide proliferation of similar research, possibly in labs that may not have well developed biosafety cultures and training. Both Fouchier and Kawaoka worked in facilities rated at 'biosafety level 3 enhanced'; but to expect that all such research would be done carefully everywhere is "utter malarkey", Caplan told the meeting.

The World Health Organization has recommended that work to make H5N1 viruses more transmissible in mammals, which flu researchers voluntarily halted in January, remain suspended until the relevant authorities have assessed the safety conditions for such research (see *Nature* **482**, 447–448; 2012). US and Dutch authorities are expected to release their verdicts within weeks, which Kawaoka said "would be the time to lift the moratorium". But others warned that doing so before broader debate, including planned hearings by US lawmakers, would be premature and could be perceived as arrogant.

The challenge for any oversight system will be to avoid discouraging important science while ensuring that work is limited to labs with appropriate safety standards, says one scientist, who criticizes the reluctance of many flu researchers to admit that such studies carry risks. "You'd have to have your head in the sand not to accept that there are some risks here." ■

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Nature's mutant flu
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Despite delays, China's GreenGen coal-fired gasification power plant in Tianjin is going ahead.

CLIMATE CHANGE

Slow progress to cleaner coal

China moves forward with demonstration power plant as United Kingdom revives carbon-capture programme.

BY JEFF TOLLEFSON AND
RICHARD VAN NOORDEN

With many of the world's nations dragging their feet on cleaning up fossil-fuel emissions, even slow progress stands out. This spring, China's state-owned Huaneng Group plans to fire up the first phase of its flagship clean-coal demonstration project, moving the country one step closer to capturing and storing the carbon it emits. Despite being more than a year behind schedule, the GreenGen coal gasification plant in Tianjin puts China at the forefront of global efforts to exploit coal resources without releasing carbon dioxide.

In 2008, leaders of the G8 group of nations called for the development of 20 large-scale projects demonstrating technologies for carbon capture and storage (CCS) by 2010, but countries have been slow to embrace the costly plants. Delays and cancellations have affected all but a handful of high-profile initiatives in Europe, the United States and Australia, whereas China, despite delays of its own, is still pushing forward to develop indigenous technologies.

"GreenGen represents both a high degree of technical sophistication and a real commitment on China's part to clean-energy

technology," says Julio Friedmann, head of the carbon-management programme at Lawrence Livermore National Laboratory in Livermore, California. "There can be no doubt that China has achieved something remarkable."

Originally estimated to cost US\$1.5 billion, GreenGen is being developed by a consortium of Chinese companies, including Huaneng, together with Peabody Energy of St Louis, Missouri. The first phase is a 250-megawatt integrated gasification combined-cycle power plant, which will convert coal into 'syngas' — a mixture of carbon monoxide and hydrogen — to be burned in specialized turbines to produce electricity. Waste carbon dioxide from these processes can be separated more easily than in conventional coal-fired power plants.

Huaneng has already begun work on a second phase — a smaller pilot plant that will send a clean stream of hydrogen through fuel cells and turbines to produce electricity, with carbon dioxide being captured for industrial use. The third phase, scheduled for 2015–20, will be a 400-megawatt power plant with full-scale carbon capture and storage in underground rock layers. That represents a substantial delay beyond the original completion date of 2015. Huaneng officials say that they revised the schedule in response to technical issues and delays to ►

► CCS projects in other nations.

GreenGen was originally seen as a follow-up to FutureGen, the flagship US initiative that was promoted and then cancelled by former president George W. Bush. In 2009, President Barack Obama revived the project, but a business consortium has yet to settle on a home for it. Many are sceptical about whether FutureGen and other US CCS projects will come to fruition, given the inability of Congress to craft a comprehensive programme for reducing greenhouse-gas emissions.

The United Kingdom is also trying to renew interest in CCS. On 3 April, the UK Department of Energy and Climate Change relaunched a £1-billion (US\$1.6-billion) competition for companies to build CCS demonstration plants. An earlier initiative, launched in 2007, fell apart in 2011 when the last remaining consortium pulled out because of concerns over costs. This time, the UK government has encouraged companies by promising that electricity produced by CCS plants can be sold at premium prices, and it will also provide £125 million for CCS research and development.

Many countries still see potential CCS plants as one-off demonstration projects, but the United Kingdom is trying to create conditions for them to become commercially viable, says Stuart Hazeldine, a geologist working on CCS at the University of Edinburgh, UK. So far, seven consortia have publicly announced their interest in the contest, which aims to commission plants in 2016–20 and store carbon dioxide in porous rock under the North Sea.

Other well-advanced initiatives include Canada's Boundary Dam Integrated Carbon Capture and Storage Demonstration Project in Saskatchewan, a 100-megawatt project to retrofit carbon-capture technology to an existing power plant; and the Texas Clean Energy Project, a 400-megawatt integrated gasification combined-cycle plant that could begin operating in 2014. Both plan to sell carbon dioxide to oil companies, which use the gas to flush oil out of reservoirs. In the Netherlands, the Rotterdam Capture and Storage Demonstration project aims to begin storing carbon dioxide in depleted gas fields under the North Sea in 2015.

Howard Herzog, a carbon-storage expert at the Massachusetts Institute of Technology in Cambridge, says that the challenge facing the entire carbon-capture industry is how to turn a profit in the absence of a serious carbon policy that rewards emissions-reduction projects financially. "The question is whether there

is a CCS market anywhere in the world," Herzog says. "If there isn't, why develop the technology?" ■

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For more about
GreenGen, see:
go.nature.com/2cmgm5

BUSINESS

Roche chases stake in medical sequencing

Biotech firm Illumina continues to resist takeover — but analysts suggest that a merger is inevitable.

BY ERIKA CHECK HAYDEN

Gene sequencing has mainly been the province of technology companies catering to researchers, but the pharmaceutical giant Roche, based in Basel, Switzerland, has other plans. It is increasing the pressure on Illumina, a DNA-sequencing company headquartered in San Diego, California, hoping to absorb the firm and use its expertise to capture the growing market in personalized medicine.

Roche proposed a merger in January, offering US\$44.50 per share, which Illumina's board rejected. On 29 March, Roche raised its bid to \$51 per share, for a total acquisition price of \$6.7 billion. Illumina's board rejected this offer, too, and adopted a 'poison-pill' provision that would give present investors the right to buy new shares at a reduced price, thereby diluting the value of Roche's shares if the deal goes through. But many observers say that the merger is all but certain. "If Roche really wants this, they are ultimately going to be able to make this go through," says John Haggerty, a mergers-and-acquisitions lawyer with Goodwin Procter in Boston, Massachusetts.

Roche's interest in Illumina stems from the latter's dominant position in genetic research; it claims that 90% of the world's sequencing is done on its machines. Roche believes that whole-genome sequencing is poised to become a lucrative diagnostic tool, and that Illumina will allow it to break into that market.

Illumina could benefit from Roche's vast sales

force, which would make it more competitive with its major rival, Life Technologies of Carlsbad, California. And Roche's experience in diagnostics and drug development could speed the translation of sequencing into the clinic, says David Ferreira, a Boston-based analyst at the investment bank Oppenheimer. Manufacturers are eager to move from next-generation sequencing technology into diagnostic technology, and "Roche could do that a lot faster" than Illumina could, he says.

Yet Illumina has fought back, arguing to its shareholders that Roche's offer undervalues the company (see 'Down and up'). Illumina, which started out in the 1990s as a microarray vendor, came to dominate the sequencing market by steadily cutting the cost and improving the speed and ease of use of its products.

However, Illumina now has numerous competitors with new platforms nipping at its heels. The firm is cagey about what technologies could replace its current sequencing machines, even as the newcomers, such as Oxford Nanopore Technologies in Oxford, UK, are promising big leaps in the speed and price of sequencing — with the aim of attaining the \$1,000 human genome. Industry observers question whether Illumina's nimbleness will survive a Roche merger. They point to Roche's 2007 acquisition of sequencing company 454 Life Sciences in Branford, Connecticut, which has since failed to live up to hopes that it would capture a significant part of the market.

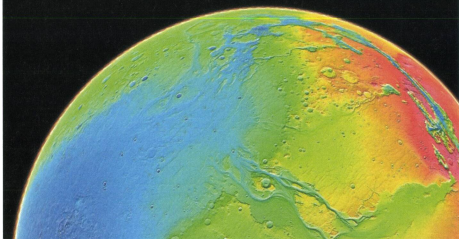
To up the pressure, Roche has nominated six people for Illumina's board of directors who, if elected by shareholders at the company's annual meeting on 18 April, might coax the board to work out a deal. But with advisory firms cautioning major shareholders to hold out, Roche will probably continue to raise its price to entice institutional investors and arbitrage traders, who bought Illumina shares expecting Roche to sweeten its offer. "It's just a matter of how much the Illumina board can force them into raising their price before the stockholders start accepting the offer," says Haggerty.

For Illumina, the end could be bittersweet. The company may be in a prime position to dominate the potentially lucrative clinical-sequencing market. But it may also see the end of its glory days as a key innovator in sequencing. ■

DOWN AND UP

Illumina's share price fell last year owing to gloom over biomedical-research funding. Roche's January bid boosted it, but only part way.





Sea change: previous ideas that lowlands on Mars (blue) once hosted oceans are being overturned.

ASTRONOMY

Dreams of water on Mars evaporate

Climate models reveal the red planet was mostly cold and dry.

BY ERIC HAND

The debate began when nineteenth-century Italian astronomer Giovanni Schiaparelli thought he saw water-filled *canali*, or channels, on the red planet: just how wet was Mars? "This is a pendulum that has been swinging back and forth," says Jeff Andrews-Hanna, a planetary scientist at the Colorado School of Mines in Golden. The *canali* were an illusion, and no one doubts that Mars today is dry except for possible meagre seeps of groundwater. But in recent years researchers have come to accept that ancient Mars had lakes or even oceans — favourable conditions for life. "This is what started off the fever for Mars science," Andrews-Hanna says.

But the pendulum is swinging again. Last month, Jim Head, a planetary scientist at Brown University in Providence, Rhode Island, threw a wet blanket on the idea that Mars was ever very wet at all, in a keynote talk at the Lunar and Planetary Science Conference in The Woodlands, Texas. Head and others are assembling a picture of a Mars that was cold and dry from the beginning, punctuated at most by short bursts of wetness. "The notion of a palm-tree-covered Mars has waned," says Stephen Clifford, a planetary scientist at the Lunar and Planetary Institute in Houston, Texas, who is organizing a conference in May on the early climate of Mars.

The first spacecraft to visit Mars, in the 1960s and 1970s, showed a bone-dry planet pocked with craters, much like the Moon. But high-resolution cameras on orbiting missions such as the Mars Global Surveyor, which launched in 1997, showed valley networks — braided and branched channels about 3.7 billion years old that seemed to have been carved by water. Then in 2005, a spectrometer on the Mars Express satellite found widespread evidence for clays¹ — minerals that testify to hundreds or even thousands of years of exposure to water. Suddenly, geologists did not seem so bold in tracing out the palaeo-shorelines of an ocean that could have covered the planet's entire low-lying northern hemisphere.

But Head and others have countered that view with three main lines of evidence. The first comes from models of the ancient Martian climate that fail to predict temperatures high enough for rain, or for liquid water to persist on the surface at all. The young Sun was fainter than it is today, and even if the young Mars had a thicker atmosphere, its greenhouse effect would probably not have warmed the planet above freezing, says François Forget at the University of Paris. He has submitted the journal *Icarus* what he says is the most sophisticated three-dimensional

climate model for Mars so far. It predicts that any water on Mars would have been bound as ice at higher elevations.

By next month's conference, Forget hopes to incorporate the effect of greenhouse gases, such as sulphur dioxide, that could have been present occasionally in huge doses. He hopes to test the idea, proposed by Head, that sulphurous bouts of volcanic activity could have warmed the atmosphere for brief periods, just enough to melt the icy highlands and unleash torrents that could have carved the valley networks. Other researchers invoke local melting from the heat from large asteroid impacts.

Closer inspection of the valley networks supports the sporadic presence of water rather than an enduring wet climate, Head says. He points to studies, including his own, showing that some of the networks are isolated geographically and in time, having formed hundreds of millions of years apart².

Even the clay minerals may not support a wet planet. A team using a spectrometer on the Mars Reconnaissance Orbiter found that roughly 80% of the clays occur together with other minerals that form at relatively high temperatures. This suggests that the clays formed not in cool surface water but underground, in water warmed by leftover heat from Mars's formation, says Bethany Ehlmann, a planetary scientist at the California Institute of Technology in Pasadena, who led the study³.

Curiosity, the NASA rover that is expected to land on 5 August, may get a closer look. A thin ring of clay encircles a 5-kilometre-high mound in the middle of the Gale crater, where the rover will touch down. Although Ehlmann says that these clays are probably among the 20% that formed with surface water, their textures will reveal something about the extent of that water. Were the clays deposited in a persistent, deep lake? Or could the waters have been shallow and short-lived? Some geologists have even suggested that the minerals could have formed in the presence of ice.

Andrews-Hanna says the shift in thinking doesn't rule out life on ancient Mars, but instead drives it deeper underground. "If Mars's climate was never stable, that would have been a challenge for life," he says. "But as you go deeper in the subsurface, things become more stable."

Head doesn't think his revisionism causes too many problems for ancient life even at the surface. He says ancient Mars could have been very much like the dry valleys and ephemeral lakes of Antarctica, where dried-up mats of algae bloom in the warmer season, nourished by a trickle of meltwater from the icy highlands above. "The public perception is that warm and wet equals life," he says. "If you look at the range of life on Earth, there's no reason to suspect that life is limited to that." ■

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For more on the red planet, see:
go.nature.com/1z1tb3

1. Poulet, F. et al. *Nature* **438**, 623–627 (2005).
2. Fassett, C. I. & Head, J. W. III *Icarus* **195**, 61–89 (2008).
3. Ehlmann, B. L. et al. *Nature* **479**, 53–60 (2011).



The Square Kilometre Array would be the world's most powerful radio telescope (artist's impression).

ASTRONOMY

Giant telescope may get two homes

Split-site solution could allow both Australia and South Africa to host parts of the Square Kilometre Array.

BY GEOFF BRUMFIELD

With the battle to host the world's most powerful radio telescope growing increasingly acrimonious, the project's leaders are considering whether to divide the spoils.

The US\$2.1-billion Square Kilometre Array (SKA) would open a window on the early Universe. As yet, international partners have not committed to covering the hefty price tag. But if the project goes ahead, it would bring a

flood of funding, prestige and scientific opportunities to one of the two competing teams: South Africa or joint bidders Australia and New Zealand.

Last month, after considering the scientific merits of the two sites, the SKA Site Advisory Committee concluded that South Africa offered marginally better opportunities (see *Nature* <http://doi.org/hst;2012>). Since then, the already-intense lobbying from both sides has increased, with politicians from each country insisting that they would prevail.

Now, the SKA management board has asked a new scientific panel to determine whether the telescope, made up of 3,000 15-metre-wide dish antennas and many more simpler antennas, could be divided between the two proposed sites.

Politicians in Australia and South Africa say that they oppose any split in the project, but John Womersley, head of the SKA board and chief executive of the UK Science and Technology Facilities Council, says that a compromise may be one way to resolve the battle. "I have heard astronomers that I respect say that such a solution is possible," Womersley told *Nature*.

Astronomers plan to use the SKA to measure radio signals at a frequency of around 1 gigahertz, probing early galaxy formation and investigating how gravity behaves near black holes. Capable of detecting a television signal from up to 15 parsecs (50 light years) away, the telescope might even aid in the search for extraterrestrial intelligence. Supercomputers will integrate the signals from the dishes and antennas, creating a virtual dish with a combined collecting area of 1 square kilometre (see *Nature* **480**, 308–309; 2011).

"Normally when you have a giant dish, you cannot split it, but the SKA has many different components," says Heino Falcke, a radio astronomer at Radboud University in Nijmegen, the Netherlands. The easiest solution would be to divide the project by placing the higher-frequency dishes on one continent and the lower-frequency antennas on the other.

Doing so would almost certainly raise the SKA's price tag, because computing centres and power would be needed in both remote locations, says Albert Zijlstra, director of the Jodrell Bank Centre for Astrophysics in Manchester, UK. But splitting the antennas by frequency would avoid the need for a high-throughput data link connecting the two sites, something that Zijlstra and Falcke both expect would be even more costly.

Zijlstra says that he can see little scientific advantage to splitting the project, aside from a few extra hours of observation time gained from having the telescope's two parts separated by such a vast distance. Falcke agrees. "It's a question of politics," he says.

The panel is expected to deliver its results by mid-May, when the SKA board will meet again to discuss the site. ■

SKA/UNIVERSITY OF ASTRONOMY PRODUCTIONS/SKA PROGRAM DEVELOPMENT OFFICE



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COMING OF AGE

Researchers in Britain have tracked thousands of children since their birth in the 1990s. Now the study is 21, and turning to the next generation.

BY HELEN PEARSON

In a secure storage barn on the outskirts of Bristol, nearly 9,000 placentas float in plastic buckets of formaldehyde.

Twenty-five kilometres away, in the basement of a university building, baby teeth from more than 4,000 children fill cardboard boxes in a walk-in freezer. Next door are some 15,000 nail clippings and 20,000 locks of hair. A few steps farther, a parade of freezers house row upon row of bar-coded blood cells, plasma, urine, saliva and chunks of umbilical cord that together make up a tissue library with more than one million entries.

This library is the harvest from an unusual study of humanity. In 1990, researchers started to collect tissues and detailed information from more than 14,500 pregnant women in this western British city and its surrounding region of Avon. The women filled in more than 100 pages of questionnaires about their health, relationships, work and home. After birth, researchers tracked the children's development through surveys, clinical examinations and biological samples. They know what the kids ate, when they first talked, how often they fell sick and when a parent read to them — or deserted them. They know when the children started to hit puberty, drink alcohol, have sex and leave home. In that wealth of data — collected at a cost of some £42 million (US\$67 million) so far — they are tracing how genetic and environmental factors in the children's early years affect their later ones.



Amy Murdoch-Davis is one of 14,000 people who have been studied since birth in and around Bristol, UK; her baby, Esmé, will soon join the study, too.

Now, the Bristol cohort study is coming of age, literally and scientifically. This spring, the first members of the group turn 21. And on 18 April, leaders of the study and their collaborators from around the world will meet in Bristol to discuss what they have learned from the Avon Longitudinal Study of Parents and Children (ALSPAC), also known as the Children of the 90s. The rich collection of tissues and behavioural data — a comprehensive phenotype for each of thousands of participants — makes the study unique and invaluable, say its leaders. "It's the deepest phenotyping and biobank resource of any large birth cohort — unequivocally," says George Davey Smith, the study's scientific director. The results have generated more than 700 scientific papers and range from policy-changing health advice for pregnant women and young children to the discovery of genetic factors involved in fetal growth, obesity, allergies and bone density¹⁻⁴. The study has also inspired and guided other birth cohorts, including the world's largest,

which is tracking more than 100,000 children in Norway.

Yet many of the data and samples remain untouched, and the cohort leaders acknowledge that the most important findings are likely to emerge years from now, some of them courtesy of new techniques that the founders of the study could barely have imagined. Davey Smith and his colleagues are just starting to analyse the children's genomes — 2,000 of which have been fully sequenced — and epigenomes, the catalogue of chemical footprints left on a child's DNA by experiences in the womb or in the world. The researchers say that such studies will help them to move from a slew of loose epidemiological associations — between a mother's fish-eating and her child's intelligence, for example — to the genetic and epigenetic players responsible for those links.

As the children themselves become parents, the team is expanding the scope of the study to a new generation. Still, at the heart of all this remains an unanswered question: how much value is there in collecting a huge amount of

ALSPAC: CHILDREN OF THE 90S



The Bristol study has banked more than 1 million samples including blood, urine, saliva and placentas.

information about human life when the scientific pay-off is unknown? Marcus Pembrey, who was director of genetics at ALSPAC until 2005, recalls that when he initially told colleagues that he wanted to measure "everything" about the children, "they laughed and said you can't study everything. And I said you have to study everything. You don't know what will be important in the future."

CONCEPTION AND BIRTH

Today, the effort to gather everything is headquartered in a concrete slab of a building in central Bristol. On the ground floor, a brightly painted annex houses a clinic where the children of the 90s come for their regular medical examinations. A few middle-aged men — the dads of the cohort members — are here, going from room to room for blood-taking, cognitive testing and bone scans. The study scientists are bringing in as many dads as they can to collect clinical data such as DNA, height, weight and blood pressure, in an effort to ramp up studies on the fathers' health, as they already have for the mothers.

Also in the clinic is 19-year-old cohort member Amy Murdoch-Davis, with her five-month-old baby Esmé. Last month, ALSPAC received provisional ethics approval to start recruiting all the children of its cohort members, an effort it hopes to launch in earnest later this year. Esmé will be one of the first signed up. "I've been a guinea pig all my life," Murdoch-Davis says, "and she can be a guinea pig too." This time around, the researchers want to collect even more samples, including breast milk and the babies' first stools.

The study itself was a struggling infant when Murdoch-Davis was born. Its leader, Jean

Golding, a mathematician turned epidemiologist, had studied stillbirths and neonatal deaths and was eager to learn how events in pregnancy and infancy affect a child's health and development. She was convinced that studying a large cohort, starting in pregnancy and gathering as much information as possible, was the best way to find answers. Funders, however, saw it differently. "Everybody thought 'if you have a cohort study you're tied down to carrying on funding it — and it's a bottomless pit,'" she says.

Golding eventually scraped together some money by writing grant applications that focused on specific diseases. She drummed up interest from mothers by talking on television and radio, and sending an army of midwives to antenatal classes and doctor's surgeries. The 14,541 pregnancies eventually included in the study encompassed more than 70% of those eligible in the region between April 1991 and December 1992. The buckets of fresh placentas started stacking up. And so did other data, such as blood squeezed from the babies' heels during the early clinic visits and reams of questionnaires filled out by the mothers (see "Building a bank of life"). Little was left unasked. Does baby drink breast milk or formula? Has he or she had antibiotics or skin ointment? Do you have a telephone; a tumble dryer; cockroaches?

Within a year or two of starting, however, the cash was draining away. Golding employed her 40–50 staff one month at a time, never

sure whether she would have enough to pay them again. And there was no time or money to analyse the data that were flooding in. "We were well in the red," Golding says. "I was just exhausted."

CHILDHOOD AND ADOLESCENCE

A few years later, just as parents were collecting baby teeth from beneath thousands of pillows and mailing them to the research team, the first data analyses started coming through. Researchers now point to a handful of results for which the cohort is famous, and that had an impact on public-health policy. One showed that eating oily fish during pregnancy was associated with better eye and cognitive development in children⁵⁶. Another helped to cement advice that babies should be put to sleep on their backs to reduce the risk of cot death, by showing that this sleeping position did not cause any developmental delays⁵⁷. A third showed the first association between peanut allergy — an emerging epidemic in Western countries — and peanut oil in baby lotions⁵⁸. Manufacturers soon started identifying the ingredient on labels.

As the children entered their second decade, the study found itself on firm financial ground for the first time. The Wellcome Trust and the Medical Research Council awarded it core funding that has totalled some £21 million for 2001–14, and investigator-led grants have brought in much more.

With the study maturing comfortably, Golding retired in 2005. ("They stopped paying me," as she puts it, still firmly ensconced in her office at the University of Bristol.) Lynn Molloy, a social scientist, took over the managerial reins, and Davey Smith, an epidemiologist passionate about genetics, grasped the scientific ones.

The study entered a productive scientific adolescence even as the cohort members suffered their own teen agonies. Questionnaire

data revealed that 19% of 16–17-year-olds cut or otherwise hurt themselves. By age 18, some 5–10% had experienced some form of psychosis — "more common than people had previously suspected," says Stanley Zammit, a psychiatrist working with cohort data at Britain's Cardiff University — even though few are likely to go on to develop schizophrenia or a related condition.

At the same time, genetics was finally entering the picture. In the early years of the project, examining the children's genotypes was just a fond hope. Scientists had announced a draft human genome in 2001, but that was a multibillion-dollar megaproject. Quite what to do with several thousand raw human genomes,

**"IT'S THE DEEPEST
PHENOTYPING AND
BIOBANK RESOURCE
OF ANY LARGE BIRTH
COHORT."**

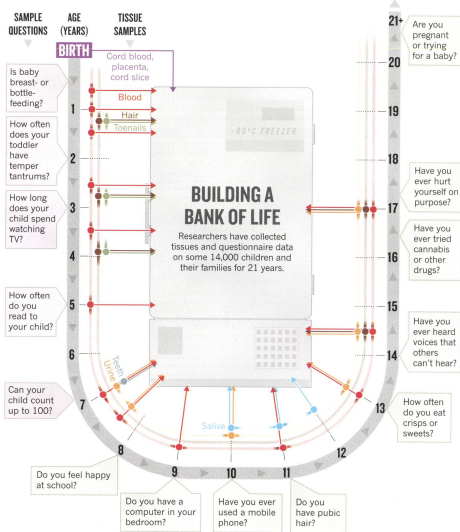
no one knew. "The interesting thing was that although all these high powered alpha-male geneticists were talking the big talk about genetics — when you actually gave them DNA for a thousand individuals it was too much. They didn't have the technology," Golding says.

In 2007, however, data from ALSPAC and several other large human biobanks were used to scour the human genome for single-letter variants associated with obesity. The work turned up a gene called *FTO*, and found that adults with two 'risk' copies of this gene are about 3 kilograms heavier, on average, than those with no risk copies⁵. The discovery became a poster child for the identification of risk alleles through genome-wide association studies, a technique that was sweeping through the genetics community at the time. The Bristol cohort — with its extensive bank of DNA and medical data — was perfectly placed to catch the wave. Its data have been used to identify genetic sequences associated with fetal growth⁶, bone density and childhood growth⁴, tooth development⁸, facial features¹⁰ and more. Researchers are now hunting for genetic links to intelligence, educational attainment and gender orientation.

Davey Smith sees other opportunities to explore associations in the cohort's DNA. He finds it frustrating that epidemiological studies often reveal a correlation between two factors — poverty and obesity, say — without proving that one causes another. The trouble is, many social and biological factors tend to correlate anyway: people who smoke also tend to drink more alcohol, eat unhealthy food, be poorer, weigh more and have high cholesterol and other signature biomarkers of ill health.

One way to filter true causes from the correlations is to compare one cohort with another, something Davey Smith did recently to find 'causal associations' with breastfeeding. In the Bristol cohort, breastfeeding is correlated with less obesity, lower blood pressure, higher intelligence and more good things besides. But in the United Kingdom, breastfeeding mothers are also more likely to be middle- or upper-class. So is it breastfeeding that helps children, or some other aspect of a comfortable life? When Davey Smith and his colleagues looked at a birth cohort based in Pelotas, Brazil, where breastfeeding is not linked to socioeconomic status, the links with obesity and blood pressure fell apart, but the link with IQ held up¹¹.

Researchers are also finding true causes through Mendelian randomization, a genetic technique that is exciting epidemiologists. Stephanie von Hinke Kessler Scholder, an economist at the University of York, UK, is using this method to unpick the associations between children's obesity and poorer performance in school. Obesity is also correlated with lower socioeconomic status, so Scholder sought to test whether obesity directly hinders performance (because of bullying, for example), whether kids who are obese do less



well because they come from disadvantaged families, or whether some other factor could explain the correlation.

Scholder took the banked DNA of the ALSPAC children and divided them up into groups on the basis of the make-up of two weight-related genes, *FTO* and *MC4R*. (Children with all 'heavy' copies of these genes weigh a few kilograms more on average than those with all 'light' copies.) Gregor Mendel's laws of inheritance ensure that the heavy and light forms of the genes are shuffled and randomly delivered to children across a population, irrespective of their social class or any other confounding factor. And when Scholder removed confounders from the equation in this way, she found that children with the heavy versions of *FTO* and *MC4R* did just as well on school tests at age 14 as those who didn't have them. Dispelling the false idea that fat children do worse at school is "a positive thing," she says¹².

The most technologically advanced answer to the causation problem sits upstairs in the Bristol headquarters of the study. There, a £300,000 machine is poised to start rapid analyses of methylation — an epigenetic mark that

controls gene activity. By looking at 450,000 sites in the cohort members' genomes, the researchers will build up a bank of methylation data on blood samples taken from the children at birth, at ages 7 and around 17, and on the mothers during pregnancy and 17 years later. "It's a unique resource," says Davey Smith.

In a study published last month¹³, Caroline Relton at Newcastle University, UK, who is leading the epigenetic work for ALSPAC, looked at methylation on an array of genes in the umbilical-cord blood of cohort children. She found that methylation signatures on nine genes at birth showed some association with body height and tissue composition at age nine. The finding hints that events during pregnancy might shape gene expression early in life — eventually resulting in altered growth and weight gain. It also "provides a flavour of the sort of thing we can address more powerfully when we get this huge data set under way," Relton says. Next, the team plans to search for methylation patterns linked to factors such as neural development, behavioural problems, cardiovascular health and fatty liver disease. The researchers will



Jean Golding, pictured here with cohort children in 2001, started the study in the early 1990s.

also look for potential causes of these methylation patterns — associations with a mother's smoking, alcohol intake, weight gain during pregnancy, folate levels and exposure to air pollution.

NEXT GENERATION

The methylation robot is just one piece of the high-tech equipment involved in the study. In one lab in Bristol, blood cells taken from the dads that day are being spun down and divided up into aliquots, ready for storage. In another, researchers are transforming cell samples into immortal cell lines. They have already banked around 7,000 such lines, which provide an endless supply of DNA and might be used for future studies of cell behaviour. Sue Ring, the head of the laboratories, says that her team take turns to carry an emergency mobile phone that will warn them of a freezer failure. After all that the participants have given to the study, she says, she feels a "duty of care for the samples".

Soon, the labs will start to process a whole new set of tissues — from the children of the cohort members. The eggs that will develop into these children were formed when their mothers themselves were babies, growing in their own mother's uterus. This means that events in the grandmothers' lives, such as an infection, stress or exposure to toxic chemicals — all recorded in the cohort database — could produce signals in the streams of data that the researchers plan to collect about

the grandchildren. "I was going to retire and wind down until we stumbled on these transgenerational events," says Pembrey, who wants to explore the third-generation consequences of traumatic events in the lives of grandparents.

With so much yet to do, has the study justified the years of effort put in so far? Cohort studies sometimes draw criticism because "they are very big and very long-term and they seem to be a lifelong source of income for investigators", says Teri Manolio, who has worked on cohorts in the past and is now director of the Office of Population Genomics at the National Human Genome Research Institute at Bethesda, Maryland.

But ALSPAC receives kudos from many researchers, including Nigel Paneth, an epidemiologist at Michigan State University in East Lansing, who runs a data-collection site for the US National Children's Study, which started recruiting in 2009. Unlike ALSPAC, the National Children's Study has ballooned into a mammoth programme with costs estimated at as much as \$6 billion, and has struggled for more than a decade to convince sceptical scientists and funders that it will pay off scientifically. Paneth says that big cohort studies can make major contributions to health if they are well planned and executed. "Can I guarantee results? Of course I can't. But I can guarantee you no results if we don't do it."

In Bristol, the team is only starting to learn the value of some of the data collected decades ago. The placentas, which had to be moved

twice, were regarded mostly as a "bloody nuisance" until three years ago, when David Barker at the University of Southampton, UK, and Oregon Health and Science University in Portland, asked to use them. Barker is famous for drawing connections between early fetal development and adult health; in the past few years he has reported that the size and shape of a placenta — the life support of the fetus — is associated with risk of adult coronary heart disease, high blood pressure and even lifespan⁶. He sent a photographer to Bristol to snap the placentas, and is now using the organs' dimensions to test some of his hypotheses. The correlations are "definitely there and amazingly strong", Barker says.

Other correlations will become clear only when the study and its participants have grown up a little more. They do, after all, have most of their lives ahead of them. Murdoch-Davis is planning the next step in hers: going to university to study English and psychology. Meanwhile, Molloy is mapping out what data to collect when the cohort members next visit the clinic, aged 24–25, when they will be close to the peak of their health. And Davey Smith has faith in future generations of scientists to find new ways to study the cohort. "My hope is that when I drop dead it's got an energetic scientific director who's implementing all these future technologies that I couldn't imagine," he says.

Perhaps, Davey Smith says, someone will be analysing data from the digital video recorders that he's thinking about handing to each expectant family, to document every significant event in their child's life. After all, storing terabytes of data doesn't cost anything — and you never know what they might one day reveal. "Digital recording allows you to store up huge banks of data for future use," he says. "It's a bit like Jean collecting the placentas. No one knew what they were going to do with them." ■

Helen Pearson is Nature's chief features editor.

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ILLUSTRATION BY JOATHAN BURTON



Bold strategies for Indian science

For a nation of its talent and education, India deserves higher scientific standing. It needs clear and honest leadership, not more money, says **Gautam R. Desiraju**.

When an Indian prime minister publicly admits that India has fallen behind China, it is news. Manmohan Singh's statement last January at the Indian Science Congress in Bhubaneswar that this is so with respect to scientific research, and that "India's relative position in the world of science has been declining", has rung alarm bells. Singh was not springing anything new on Indian scientists; many of us will admit that things are not well¹. Recognizing the problem is the first step towards reversing this slide.

At present, India has a trickle-down strategy, in which elite institutions are

supported in the hope that good science there will energize the masses, and a bottom-up approach, in which the general public is targeted with schemes to popularize science.

These approaches have converged with the setting up in recent years of tens of new universities, institutes and centres of higher learning, even though many hundreds more are desirable for a country of India's size. Although there was, curiously, no increased allocation to science in this year's Indian budget, there is hope that, as the prime minister has declared, things would improve if government support were increased to 2% of the gross domestic product (it now stands

at 0.9%). But it is a haphazard plan, with no hint of new strategies. The assumption is that the answer to our problems lies simply in more money.

As someone who has worked in India for 34 years, I am impatient with our slow progress². At the glitzy level, we have had no Nobel prize winner since C. V. Raman in 1930, no highly Shanghai-ranked university, no miracle drug for a tropical disease and no sequencing of the rice genome. At the industrial level, there have been no breakthroughs to rival the telephone, the transistor or Teflon. At the organizational level, we do not have a postdoctoral ▶

► system worth its name, and our undergraduate teaching system is in a shambles. We figure occasionally in the best journals, yet we tolerate plagiarism, misconduct and nepotism. And yet, the innate abilities and talents of India are palpable. Why is it that this country has not been able to harness its strengths into deliverables?

Money is not the primary constraining factor in our problems, nor will an abundance of it solve them. More money is undoubtedly better, but if there are deep cultural and social problems, extra money will simply drain away. The rate of any improvement will not match the rate of increase in investment*. Big problems in big countries usually emanate from a small number of core reasons. An understanding of these reasons in the context of Indian science should stem from an appreciation of the country's historical, economic and sociological profile.

It is not enough for the prime minister to resort to platitudes by saying (as in his recent speech) that "things are changing but we cannot be satisfied with what has been achieved", or that we should make "scientific output more relevant". He and his advisers must ask themselves if there are underlying causes for this lack of satisfaction and relevance. Until then, no amount of bankrolling, populism, bureaucrat bashing or whistle-stop tours by prominent Western scientists will help.

A FEUDAL MINDSET

Two aspects of the Indian psyche are particularly troubling for a country seeking its rightful place in the modern world. Our cultural value system, backed by Hindu scriptural authority, has created a strongly feudal mindset among Indians. Centuries of servitude, right up until 1947, have made the average Indian docile, obedient and sycophantic. "Behave yourself and be rewarded," is the pragmatic mantra. I believe this feudal-colonial mentality has had far-reaching and debilitating consequences for research.

The first is our lack of the ability to question and dissent that is so essential to science. Most of the faculty in our better institutions have done postdoctoral work in a foreign laboratory of consequence. Unlike young scientists in advanced countries, however, newly returned Indian lecturers typically relive their golden moments as postdocs throughout their research careers. The best research papers from India may be competent, but they do not inspire or excite. Very few Indian scientists are known as opinion-makers, trend-setters or leaders. They follow obediently.

Another consequence of this feudal mindset is our unquestioning acceptance — bordering on subservience — to older people. In this part of the world, age is blindly equated with wisdom, and

youth with immaturity. This facilitates the continuance of the status quo. Geriatric individuals with administrative and political clout reinforce their positions so well that we are unable to eject them. So we hail scientists in their eighties, film actors in their seventies and cricketers in their forties.

VARIANTS OF CORRUPTION

In healthy organizational hierarchies, the decision-makers are also active participants who have a stake in the future. We will have come of age only when Indian universities are allowed to appoint their own vice-chancellors, and institutes and national laboratories their own directors, rather than suffer the choices made by conclaves of old men in New Delhi.

The most important decisions in an academic system concern the appointment of faculty. This procedure is flawed in India. For a start, selection committees consist mostly of outsiders, and representation from within is often restricted to institutional and departmental heads. In the smaller state universities, all sorts of irregularities occur in the name of caste-based reservations. In the more influential central institutions, appointments are often made incestuously, with students of a few senior researchers filling a disproportionately large number of vacancies, or with plain academic 'inbreeding'. A good dose of regional parochialism completes the picture.

Corruption need not take a monetary form; in a national laboratory it can mean acquiescing to the notion that one's administrative head is also one's scientific superior. By that logic, and given our civilian-based system of administration, the secretaries in the science ministries in Delhi should be our most creative scientists.

These variants of corruption — along with general indifference, absence of incisive introspection, old-boys' networks, administrative vindictiveness, vagaries in research funding and studied silences — conspire to create an atmosphere that lacks innovation and creativity. Impact factors and *h*-indices become the sole arbiters of scientific excellence in such an environment. If policymakers are ignoring cultural parameters, scientists are looking only at numerical parameters.

The true measures of a country's scientific strength are found in the numbers of competent teachers and lively students in schools and undergraduate colleges, because these translate into real gains in the future. Fluffy factors, such as the numbers of

articles in *Nature* and *Science*, do not tell the real story. As a chemist, I would say that it is better to move deliberately and confidently towards the thermodynamic minimum (a system's most stable state, which has the lowest energy but is not always the easiest to achieve) rather than flit anxiously between any number of metastable kinetic states (which are easy and fast to access but have higher energies).

A large country with a well developing economy can afford this long-term strategy and vision. China need not be a comparison point* — India is endowed enough to seek its own solutions for its problems*.

THE WAY FORWARD

I suggest that our policy-makers consider the following. First, provide modest funding to a very large number of small, single-investigator, blue-sky projects — including those in state universities — to achieve a critical density of ideas and a feeling of mass participation and enthusiasm.

Second, provide heavy and directed funding into a few specific projects of national importance — such as energy, water and public health — with high levels of accountability and proper exit options. Third, reduce or abolish the present system of awards, prizes and recognitions in higher-level science. This would dissuade younger scientists from chasing awards rather than doing good science, and it would reduce the influence of the cliques who allocate prizes.

To reach a stable solution, we can employ longer-term measures that include modification or removal of caste-based quotas and reservations in the educational and research sectors; improvement of undergraduate teaching institutions and teaching laboratories with respect to greater uniformity and transparency; and clear identification of paths towards scientific and administrative growth for individuals.

Money is neither the cause nor the solution to our problems, although it can facilitate progress in an otherwise healthy climate. What is lacking in India is the quality of leadership and the level of honesty that are required for a breakthrough. When will this country see another C. V. Raman? ■

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Rapid economic growth means that the air in some Chinese cities, such as Beijing, contains more fine particles than the World Health Organization recommends.

Cleaning China's air

To reduce airborne soot, organics and sulphates, tailored strategies for each must be established and coal use limited, say **Qiang Zhang, Kebin He and Hong Huo.**

On 29 February this year, China's State Council approved its first national environmental standard for limiting the amount of fine particles in air that measure less than 2.5 micrometres in diameter. It requires the country to implement the World Health Organization's recommended interim target of an annual average of 35 micrograms per cubic metre ($\mu\text{g m}^{-3}$) for such particles by the end of 2015.

Fine particles — including soot, organics and sulphates — have a severe effect on human health and are implicated in climate change. They are emitted by combustion and industrial processes, and formed from the reactions of gaseous pollutants. If implemented properly, China's air-quality standard would have far-reaching benefits: as well as protecting human health, it would reduce air and mercury pollution in the Northern Hemisphere and slow global warming.

Achieving this goal will be a challenge. Some Chinese cities currently have fine-particle concentrations that are well above the proposed standard: levels of more than $100 \mu\text{g m}^{-3}$ have been reported¹. To meet the ambitious air-quality limits, China will have to overcome two major hurdles: its relentless increase in fossil-fuel use, which quickly wipes out any efforts to reduce emissions, and its decentralized system of environmental enforcement, which gives

undue influence to local officials who favour economic development.

Controlling air quality in China will address global environmental issues. For example, pollutants from east Asia that travel across the Pacific increase ozone concentration in the western United States². This could be relieved if China reduces emissions of nitrogen oxides (NO_x), which are precursors of fine particles and ozone. Cross-border pollution by airborne particles would similarly be reduced by cutting China's emissions of sulphur dioxide (SO_2). The use of technologies such as desulphurization, selective catalytic reduction or electrostatic precipitators to reduce Chinese emissions of SO_2 , NO_x and fine particles, respectively, can remove global pollutants such as mercury, which is released by the burning of coal.

Limiting particle pollution will also affect drivers of climate change — but not always for the better. On the one hand, reducing soot emissions by cutting coal use or using cleaner stoves will lessen radiative forcing and thus limit warming, benefiting both the climate and public health³. A stricter emissions standard for diesel vehicles, which produce soot, is another win-win solution⁴. On the other hand, reductions in SO_2 emissions from power plants would reduce atmospheric sulphate concentrations, thereby increasing radiative forcing, which has a short-term

detrimental effect on climate⁵. Thought is therefore needed as to how the various pollutants and sources should be best controlled, and a multi-pollutant abatement strategy must be developed.

A CLIMATE CHALLENGE

The control of air pollution in China is in a race with the economy. The country has maintained an annual economic growth rate of more than 8% for years, largely through the energy-intensive construction of infrastructure such as highways, railways and cities. Between 2005 and 2010, China increased its thermal-power generation by 63%, pig-iron and cement production by 74% and 76%, respectively, and vehicle production by 220% (ref. 5).

Although China has made tremendous efforts to limit air pollution, such as requiring coal-fired power plants to install flue-gas desulphurization systems and strengthening vehicle-emissions standards, these measures have not kept up with the growth of its economy and fossil-fuel use. We estimate that new equipment reduced SO_2 emissions from China's power plants by 1.5 million tonnes in 2005 and by 17.5 million tonnes in 2010 — 54% of the country's total SO_2 emissions in 2005 (32.3 million tonnes). But nationwide, total SO_2 emissions only decreased by 11% (to 28.7 million tonnes in 2010) because

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those from other sectors grew (see 'China's emissions battle'). Coal usage rose by 44% (955 million tonnes), more than one-third of which was consumed by industrial facilities (such as iron, steel and cement works) that have no desulphurization systems.

The low priority given to environmental protection and the lack of cooperation among various government agencies also hampers air-quality control. China's Ministry of Environmental Protection (MEP) manages pollutant discharge, but it is a weak player within the government system. Its decisions are often obstructed for economic reasons. For instance, in late 2011, Chinese oil companies caused a delay in the planned 2012 implementation of a stricter vehicle-emissions standard (equivalent to Europe's Euro IV standard) because they were unable to provide the necessary low-sulphur oil. Any delay is a big strike against the environment, particularly as vehicle emissions continue to rise.

Such stories are rife in local governments, which like to promote heavy industry to stimulate regional economies. Local environmental agencies are often forced to back these projects just because they are affiliated to local governments. Yet, pushed by the public, the willingness and enthusiasm of China's government for curbing air pollution has never been so strong. It is a golden opportunity for

the nation to make a change — to free itself from the trap between economic development and environmental pollution.

CONSTRAIN COAL

Because China will continue to rely on fossil fuels for the next 20 years, the government should change its thinking. Instead of trying to use more energy to ensure economic growth regardless of the consequences, it should promote development with constrained fossil-fuel use. A cap could be set for national total coal consumption, and economic plans developed under this constraint. Otherwise, emissions from increased energy use will offset any gains from emissions control. Tertiary service industries and high-technology projects could be promoted instead of energy-intensive ones.

Greater authority should be given to environmental agencies at various levels of government. The MEP should be granted more power to implement its policies and enforce regulations. A vertical administrative structure would ensure that local environmental agencies report directly to the MEP.

The impact on the global environment should be considered when formulating China's air-quality strategies, and balanced plans should be developed for each pollution source. As well as reducing SO_2 emissions, the government should endorse measures to limit soot. Controlling emissions from diesel vehicles should be a priority, and oil companies should be brought into accordance with environmental standards. For future facilities that will control SO_2 and NO_x emissions, the government should equip them with specific technologies to remove mercury, such as activated carbon injection.

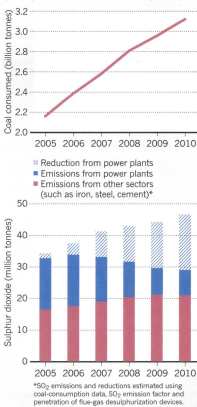
Addressing air pollution in China is a unique platform for researchers in atmospheric chemistry. Many scientific issues — such as secondary organic aerosol formation — remain to be explored. Practical control technologies for ultrafine particles and volatile organic compounds must be developed. Multinational collaboration is urgently needed; the government should make funds available to bring outstanding international scientists to China to help combat its air-pollution challenges. We all stand to benefit. ■

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CHINA'S EMISSIONS BATTLE

Rising coal use (top) has increased emissions of sulphur dioxide from some sectors, even though power plants emit less than in 2005 (bottom).



SOURCE: CHINA ENERGY STATISTICAL YEARBOOK



Q&A Michael Frayn

The playful dramatist

Author and playwright Michael Frayn explores the wellsprings of creativity through farce, philosophy and the history of science. His eclectic output ranges from non-fiction books such as *The Human Touch* (2006) to plays including *Noises Off* (1982) and *Copenhagen* (1998) — which explores the 1941 meeting between quantum physicists Niels Bohr and Werner Heisenberg, with Frayn imagining their discussions on the morality of working on nuclear weapons. With his latest novel, *Skios*, coming out next month, he talks about determinism and the paradox of existence.

What is *Skios* about?

It is a farce about an institute on a Greek island that has invited a lecturer to talk about the organization of science. The wrong lecturer shows up. I light-heartedly touch on determinism — the old question of whether human contributions to events are predetermined or whether they can't be understood in the context of causality. My view is the latter. There are two interesting things about farce. One is seeing human beings reduced to the level of machines, unable to control situations. The other is seeing people desperately thinking of ways to cope with difficult situations, inventing lies that they hope will get them out of the difficulties they're in, and of course making their difficulties worse.

Are you poking fun at the idea that human thought can be organized?

A tiny bit. People are endlessly surprised by the imagination. I'm struck by something that comes into *Copenhagen*: how physicists Otto Frisch and Rudolf Peierls came to understand how little fissile material you need to make a

nuclear chain reaction. It was because they were messing around. Everyone assumed that you would need tonnes, and there wasn't a chance of producing enough. One of the pair realized that fission had been observed only with slow neutrons, but we might want a formula that covered neutrons of all speeds. And he worked one out. Then the other one said, supposing we did have as much fissile material as we wanted, how much would be needed? And he applied the formula and discovered that it would be a matter of kilograms. These researchers weren't addressing the specific problem of building a bomb — they were working off the tops of their heads.

Why did you start writing about science?

I studied philosophy at university, and couldn't help but come across scientific questions, particularly in connection with



Skios
MICHAEL FRAYN
Faber & Faber/
Metropolitan Books:
2012. 224 pp.
£15.99/\$25

quantum mechanics. I had always had a faint idea of Heisenberg and Bohr's work, but never thought of writing about it until I read *Heisenberg's War* by Thomas Powers. Why did Heisenberg go to Copenhagen in 1941, and what were his motives in working on the German nuclear programme? Was he actually trying to build a bomb? Although there is obviously no parallel between this uncertainty and Heisenberg's uncertainty principle in quantum mechanics, there is a similar impossibility of ever reaching beyond a certain point. The result was *Copenhagen* — a play about epistemology that happens to be played out in terms of science.

Can drama teach science?

I don't think the theatre is a very good medium for explaining complex ideas. No one ignorant of nuclear physics would come out of *Copenhagen* thinking that they understood it.

In *The Human Touch*, you write about how the mind constrains our understanding of the world.

It is a paradox: we know perfectly well that we're irrelevant to the process of the

A. DALMAU/EPN, CORBIS

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► Universe — but there is nothing we can say about the Universe except in terms of what we see and think. I'm not suggesting that we make it all up arbitrarily. We're constrained by something, but it is extremely difficult to say what it is.

Some scientists would argue with that.

I can see how resistant scientists are to that side of the paradox. I was invited to CERN near Geneva, Switzerland, to talk about *The Human Touch*, and it was really daunting. They had appointed a jury that asked detailed questions. One of the jury members said beforehand: "We're going to haul you over the coals." It seemed to me — although they were all very charming and friendly about it — that they were unreconstructed Platonists. They believed that numbers and the laws of science are objective entities, whereas I think that they are constructs that we place on the world to understand it.

As a non-scientist, are you confident in writing about science?

Fortunately, professional science writers and scientists have made enormous efforts to get through to lay audiences. But people like the physicist Richard Feynman insist that if you haven't got mathematics you're never really going to understand physics — it is like trying to explain music to the tone-deaf. I made a lot of mistakes writing *Copenhagen*, in spite of getting the text read. I got letters from scientists pointing out basic errors. But I was struck by their generous tone.

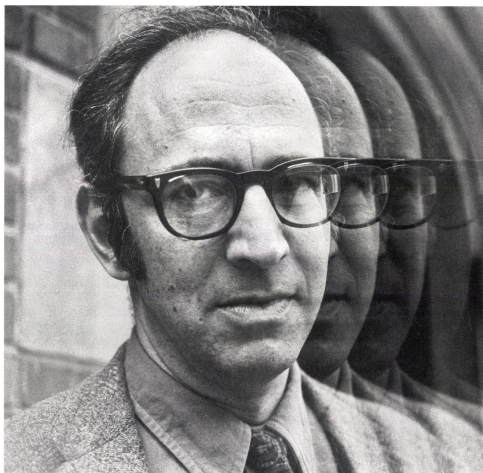
How do you approach writing?

As a writer, you can't think, "I'd like to write a play about stem-cell research and there will be these characters." It doesn't work like that: ideas just seem to fall into your head out of nowhere, and develop of their own accord. So there is resonance with the case of Peierls and Frisch, or the chemist August Kekulé dreaming about the structure of the benzene ring. There is an unconscious leap, a synthesis, that goes on, even though much science is about trying to find a specific answer to a specific problem.

So playwrights run experiments too?

Plays are called plays for a good reason. As a playwright, you are saying, what if we had enough uranium-235, or what if somebody discovered that their brother was their father, and you take over from these fictitious situations. It is messing around, but messing around often has serious results. ■

INTERVIEW BY RICHARD VAN NOORDEN



Thomas Kuhn recognized the importance of revolutionary changes, or 'paradigm shifts', in science.

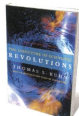
IN RETROSPECT

The Structure of Scientific Revolutions

David Kaiser marks the 50th anniversary of an exemplary account of the cycles of scientific progress.

Fifty years ago, a short book appeared under the intriguing title *The Structure of Scientific Revolutions*. Its author, Thomas Kuhn (1922–1996), had begun his academic life as a physicist but had migrated to the history and philosophy of science. His main argument in the book — his second work, following a study of the Copernican revolution in astronomy — was that scientific activity unfolds according to a repeating pattern, which we can discern by studying its history.

Kuhn was not at all confident about how *Structure* would be received. He had been



The Structure of Scientific Revolutions: 50th Anniversary Edition
THOMAS S. KUHN (WITH AN INTRODUCTION BY IAN HACKING)
Univ. Chicago Press: 2012.
264 pp. \$45, £29

denied tenure at Harvard University in Cambridge, Massachusetts, a few years before, and he wrote to several correspondents after the book was published that he felt he had stuck his neck "very far out". Within months,

Books in brief



Experiment Eleven: Deceit and Betrayal in the Discovery of the Cure for Tuberculosis

Peter Pringle BLOOMSBURY 288 pp. £18.99 (2012)

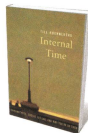
The 1943 discovery of a drug treatment for tuberculosis did much to kick-start big pharma. But this is a knotted tale, deftly unpicked by investigative journalist Peter Pringle. We learn that Albert Schatz, a US graduate student, found streptomycin in the eponymous 11th experiment on a farmyard bacterium — but that his research director, Selman Waksman, took the credit, along with patent royalties and a Nobel prize. A chance rediscovery brought Schatz the reputation he deserves.



The Forever Fix: Gene Therapy and the Boy Who Saved It

Ricki Lewis ST MARTIN'S 336 pp. \$25.99 (2012)

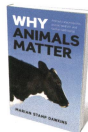
This popularized examination of gene therapy hinges on a breakthrough case: Corey Haas's recovery from Leber's congenital amaurosis type 2, which had made him virtually blind at the age of eight. Medical writer Ricki Lewis interweaves science, the history of medical trial and error, and human stories. The contrast can be intense, running from the death in 1999 of teenager Jesse Gelsinger, from a reaction to gene therapy intended to combat his liver disease, to radical successes in some children with adenosine deaminase deficiency.



Internal Time: Chronotypes, Social Jet Lag, and Why You're So Tired

Till Roenneberg HARVARD UNIVERSITY PRESS 288 pp. \$26.95 (2012)

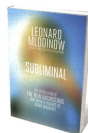
Time really is of the essence, says medical psychologist Till Roenneberg. By neglecting our body clocks — which rarely run in synchrony with the crazily cranked-up pace of modern life — we can develop "social jetlag", endangering our health and careers. Roenneberg has built his book on decades of research in everything from fungi and single-celled organisms to humans. In brilliantly minimalist terms, he explains the temporal mismatches behind teen exhaustion, early birds and night owls, and sleep phobia.



Why Animals Matter: Animal consciousness, animal welfare, and human well-being

Marian Stamp Dawkins OXFORD UNIVERSITY PRESS 224 pp. £16.99 (2012)

Too little science and too much anthropomorphism have made our approaches to animal welfare a shambles, says ethologist Marian Stamp Dawkins. Her radical rethink involves linking their welfare with our own to harness a powerful driver of change: human self-interest. Dawkins advises sidestepping the question of animal consciousness to focus on animal health and hard-wired "wants" such as foraging, to benefit both groups. Also key is never letting up on research into our intertwined existences, she says.



Subliminal: The Revolution of the New Unconscious and What It Teaches Us About Ourselves

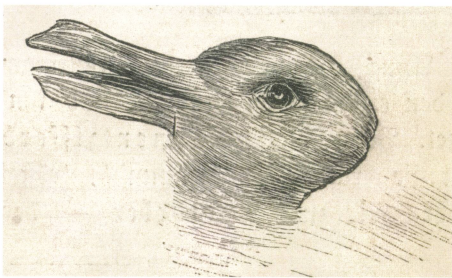
Leonard Mlodinow PANTHEON 272 pp. \$25.95 (2012)

Perception "here below the threshold of consciousness", as Carl Jung put it, is here pushed into the limelight. Physicist Leonard Mlodinow shows how humans have "parallel tiers" of a conscious brain superimposed on an unconscious mind. Drawing on research and anecdotes, Mlodinow explores the pattern-matching, gap-filling role of the unconscious in perception, memory, sociality, emotions and self-estimation. An illuminating journey through a hidden world.

however, some people were proclaiming a new era in the understanding of science. One biologist joked that all commentary could now be dated with precision: his own efforts had appeared "in the year 2 B.K.", before Kuhn. A decade later, Kuhn was so inundated with correspondence about the book that he despaired of ever again getting any work done.

By the mid-1980s, *Structure* had achieved blockbuster status. Nearly a million copies had been sold and more than a dozen foreign-language editions published. The book became the most-cited academic work in all of the humanities and social sciences between 1976 and 83 — cited more often than classic works by Sigmund Freud, Ludwig Wittgenstein, Noam Chomsky, Michel Foucault or Jacques Derrida. The book was required reading for undergraduates in classes across the curriculum, from history and philosophy to sociology, economics, political science and the natural sciences. Before long, Kuhn's phrase "paradigm shift" was showing up everywhere from business manuals to cartoons in *The New Yorker*.

Kuhn began thinking about his project 15 years before it was published, while he was working on his doctorate in theoretical physics at Harvard. He became interested in ▶



The duck-rabbit figure shows how two pictures can be derived from the same evidence.

► developmental psychology, avidly reading works by Swiss psychologist Jean Piaget about the stages of cognitive development in children.

Kuhn saw similar developmental stages in entire sciences. First, he said, a field of study matures by forming a paradigm — a set of guiding concepts, theories and methods on which most members of the relevant community agree. There follows a period of “normal science,” during which researchers further articulate what the paradigm might imply for specific situations.

In the course of that work, anomalies necessarily arise — findings that differ from expectations. Kuhn had in mind episodes such as the accidental discoveries of X-rays in the late nineteenth century and nuclear fission in the early twentieth. Often, Kuhn argued, the anomalies are brushed aside or left as problems for future research. But once enough anomalies have accumulated, and all efforts to assimilate them to the paradigm have met with frustration, the field enters a state of crisis. Resolution comes only with a revolution, and the inauguration of a new paradigm that can address the anomalies. Then the whole process repeats with a new phase of normal science. Kuhn was especially struck by the cyclic nature of the process, which ran counter to then-conventional ideas about scientific progress.

At the heart of Kuhn's account stood the tricky notion of the paradigm. British philosopher Margaret Masterman famously isolated 21 distinct ways in which Kuhn used the slippery term throughout his slim volume. Even Kuhn himself came to realize that he had saddled the word with too much baggage; in later essays, he separated

his intended meanings into two clusters. One sense referred to a scientific community's reigning theories and methods. The second meaning, which Kuhn argued was both more original and more important, referred to exemplars or model problems, the worked examples on which students and young scientists cut their teeth. As Kuhn appreciated from his own physics training, scientists learned by immersive apprenticeship; they had to hone what Hungarian chemist and philosopher of science Michael Polanyi had called “tacit knowledge” by working through large collections of exemplars rather than by memorizing explicit rules or theorems. More than

most scholars of his era, Kuhn taught historians and philosophers to view science as practice rather than syllogism.

Most controversial was Kuhn's claim that scientists have no way to compare concepts on either side of a scientific revolution. For example, the idea of “mass” in the Newtonian paradigm is not the same as in the Einsteinian one, Kuhn argued; each concept draws meaning from separate webs of ideas, practices and results. If scientific concepts are bound up in specific ways of viewing the world, like a person who sees only one aspect of a Gestalt psychologist's duck-rabbit figure, then how is it possible to compare one concept to another? To Kuhn, the concepts were incommensurable: no common measure could be found with which to relate them, because scientists, he argued, always interrogate nature through a given paradigm.

Perhaps the most radical thrust of Kuhn's analysis, then, was that science might not be progressing toward a truer representation of

the world, but might simply be moving away from previous representations. Knowledge need not be cumulative: when paradigms change, whole sets of questions and answers get dropped as irrelevant, rather than incorporated into the new era of normal science. In the closing pages of his original edition, Kuhn adopted the metaphor of Darwinian natural selection: scientific knowledge surely changes over time, but does not necessarily march towards an ultimate goal.

And so, 50 years later, we are left with our own anomaly. How did an academic book on the history and philosophy of science become a cultural icon? *Structure* was composed as an extended essay rather than a formal monograph: it was written as an entry on the history of science for the soon-to-be-defunct *International Encyclopedia of Unified Science*. Kuhn never intended it to be definitive. He often described the book (even in its original preface) as a first pass at material that he intended to address in more detail later.

To me, the book has the feel of a physicist's toy model: an intentionally stripped-down and simplified schematic — an exemplar — intended to capture important phenomena. The thought-provoking thesis is argued with earnestness and clarity, not weighed down with jargon or lumbering footnotes. The more controversial claims are often advanced in a suggestive rather than declarative mode. Perhaps most important, the book is short: it can be read comfortably in a single sitting.

For the 50th-anniversary edition, the University of Chicago Press has included an introductory essay by renowned Canadian philosopher Ian Hacking. Like Kuhn, Hacking has a gift for clear exposition. His introduction provides a helpful guide to some of the thornier philosophical issues, and gives hints as to how historians and philosophers of science have parted with Kuhn.

The field of science studies has changed markedly since 1962. Few philosophers still subscribe to radical incommensurability; many historians focus on sociological or cultural features that received no play in Kuhn's work; and topics in the life sciences now dominate, whereas Kuhn focused closely on physics. Nevertheless, we may still admire Kuhn's dexterity in broaching challenging ideas with a fascinating mix of examples from psychology, history, philosophy and beyond. We need hardly agree with each of Kuhn's propositions to enjoy — and benefit from — this classic book. ■

David Kaiser is Germeshausen Professor of the History of Science at the Massachusetts Institute of Technology in Cambridge. His latest book is *How the Hippies Saved Physics* (Norton, 2011). e-mail: dkaiser@mit.edu

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Correspondence

Primate studies: hear the public's views

A painful irony in the disrupted flow of primates to US research labs (*Nature* 483, 381–382; 2012) is that the number being used in laboratory experiments is at an all-time high.

If the scientific community is to maintain the support and trust of the public, which funds much of its work, then research practices and policies should change to reflect society's views on what constitutes the ethical treatment of animals. These changes need to be speeded up.

Committees that review and approve animal experiments at US facilities should not be dominated by those who work in animal labs and have vested interests in continuing animal research (L. A. Hansen et al. *Animals* 2, 68–75; 2012). There are too few members of the public on these US committees, and those who are involved say that they are often marginalized. In other countries, such as Sweden and Australia, half or one-third of committees must comprise non-scientists and animal-welfare representatives.

If scientists continue to disregard the substantial and growing public opposition to harmful research on primates and other animals, more protest campaigns are inevitable.

Lawrence A. Hansen *University of California, San Diego, La Jolla, California, USA.*
lahansen@ucsd.edu

Primate studies: fix welfare issues first

Record numbers of non-human primates are being used in US labs, so it is unlikely that limiting imports will hold back vital areas of research as you imply (*Nature* 483, 381–382; 2012).

A report from the American Anti-Vivisection Society (AAVS; see go.nature.com/gbqlcl) indicates that imports of monkeys born to wild-caught

parents quadrupled during 1998–2008. Conservationists are concerned about global trade in crab-eating macaques (*Macaca fascicularis*), the import of which has doubled in recent years.

Scientists must urgently address the extreme animal-welfare issues surrounding these imports. The AAVS report, which is based on information from US federal agencies and scientific studies, has revealed that monkeys destined for US labs typically endure long, gruelling air and land transportation; entire groups have been killed after quarantine on testing positive for tuberculosis; many die from transport injuries or stress in quarantine; and survivors show negative physiological and behavioural effects for several months after the journey.

More airlines are likely to back away from a dirty job that they are ill-equipped to do properly. **Crystal Miller-Spiegel** *American Anti-Vivisection Society, Jenkintown, Pennsylvania, USA.*
cmillerspiegel@aavs.org

Primate studies: trials don't always translate

In your discussion on the campaign against animal research (*Nature* 483, 373–374; 2012), you mention a study in macaques that has moved into early clinical trials in humans, with promising results. Sadly, there is a yawning chasm between early promise in trials and efficacy.

The US Food and Drug Administration reports that more than 90% of trials fail (see go.nature.com/h2365q), even though the treatments tested, by definition, were considered sufficiently efficacious and safe in animals to merit a clinical licence.

Many other treatments to protect the brain after stroke have failed in humans, despite success in rodent and primate trials (V. E. Collins et al. *Ann. Neurol.* 59, 467–477; 2006). None of the 85 or so candidate HIV vaccines

that were effective in primates has so far worked in humans (J. Bailey *Altern. Lab. Anim.* 36, 381–428; 2008). The monoclonal antibody that caused severe inflammatory reactions in a 2006 clinical trial at Northwick Park Hospital, London, caused no problems in primates at 500 times the dose given to the human volunteers.

The public is rightly concerned about the transportation of primates for questionable experimental purposes. These cannot justify the degree of suffering involved during capture, in breeding and holding facilities and during lengthy transportation (see go.nature.com/sbvuvj).

Michelle Thew *British Union for the Abolition of Vivisection, London, UK.*
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Higgs can claim name of massive boson

Attempts to rule against naming the Higgs boson after physicist Peter Higgs suggest that political correctness is taking over from scholarship (*Nature* 483, 374; 2012). Your suggestion that the name Higgs should be retained for reasons akin to business branding is hardly better. Higgs has a unique claim to the massive boson in question.

My book *The Infinity Puzzle* (Oxford Univ. Press, 2011) covers the history of the Higgs hypothesis in detail. It is true that Higgs is one of several theorists who, in 1964, independently discovered how to give mass to fundamental particles, and that it would be inappropriate to refer to the hypothesis of mass generation as the 'Higgs mechanism'. However, it was Higgs alone who drew attention to the massive boson whose detection can prove the hypothesis. So naming the boson after him, as Ben Lee did in 1972, is justifiable.

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More credit due to India's scientists

Any increase in India's science budget for 2012–13 is likely to be wiped out by a 5–10% rise in the cost of research commodities, owing to the country's high rate of inflation (*Nature* 483, 384; 2012). Neither will the modest extra funding tackle the glut of unemployed science PhD graduates (for example, around 60% of female science PhDs do not have a research position).

The reasons for this situation are not just economic. In my opinion, India's policy-makers are failing to recognize scientists' achievements. In May last year, for example, environment minister Jairam Ramesh intimated that India's elite institutions, which include the Indian Institutes of Technology and of Management, fall short of world-class standards; the head of the prime minister's scientific advisory council, C. N. R. Rao, seems to agree with this view (see go.nature.com/snnbtt).

Yet India is ranked 11th in the world by number of publications and 16th on the basis of total citations during the past 10 years. Many of these publications were in leading international journals. Instead of squandering this talent, the government should provide the incentive and the means for the nation to fulfil its potential. **Jagadeesh Bayry** *Institut National de la Santé et de la Recherche Médicale, Paris, France.*
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CONTRIBUTIONS

Correspondence may be sent to correspondence@nature.com after consulting the author guidelines at go.nature.com/cmchno. Alternatively, readers may comment online: www.nature.com/nature.

F. Sherwood Rowland

(1927–2012)

Atmospheric chemist who linked human activity to ozone depletion.

Chlorofluorocarbons (CFCs) were a triumph of the chemical industry and a mere curiosity in atmospheric science when Sherwood (Sherry) Rowland, with his postdoc Mario Molina, recognized in 1973 that these seemingly inert gases posed a threat to Earth's ozone layer. Returning home one evening, Rowland remarked to his wife Joan that the research "is going very well, but it may mean the end of the world".

In their laboratory at the University of California, Irvine, Molina and Rowland had discovered that CFC-11 (CFCl_3) and CFC-12 (CF_2Cl_2), then widely used as refrigerants and aerosol propellants, readily absorbed ultraviolet light and broke down to release reactive chlorine. This work was the first step in tracing the causal chain linking industrial production of CFCs with global ozone depletion — and won Rowland and Molina the 1995 Nobel Prize in Chemistry, shared with Dutch chemist Paul Crutzen.

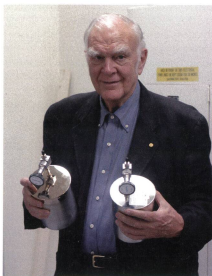
Surrounded by his family at his home in Corona del Mar, California, Rowland died on 10 March, aged 84, from complications of Parkinson's disease. He was born in Delaware, Ohio; his mother was a Latin teacher and his father taught mathematics at Ohio Wesleyan University in Delaware, where Rowland attended college after graduating from high school at 15. When he was old enough, he enlisted in the US Navy. As a lanky athlete, he readily found a home in sports teams in the Navy and later in graduate school at the University of Chicago, Illinois, where he played baseball for the university and for a semi-professional team.

Rowland earned his PhD in nuclear chemistry at Chicago under chemist Willard Libby and was taught by four other faculty members; counting Sherry, all six would later receive Nobel prizes. He met Joan at Chicago, and they moved to take up his early jobs at Princeton University in New Jersey and at the University of Kansas in Lawrence. In 1964, Rowland accepted an offer to start up the chemistry department at a new University of California campus in the then-unbuilt city of Irvine. Later, with atmospheric chemist Ralph Cicerone, he also helped to found the Earth system science department.

The elegance of Molina and Rowland's 1974 *Nature* paper remains impressive to today's atmospheric chemists, who live in a world of satellite observations and supercomputers. Stratospheric chemistry at the time was based on balloon-borne samplers of

trace gases and on one-dimensional models that could now easily run on a smart phone.

Nonetheless, the pair measured the ultraviolet cross sections of CFC-11 and -12 in the lab, calculated their photolytic destruction rates in the atmosphere and derived their atmospheric lifetimes as 50–100 years. They reviewed industrial production and emission of CFCs, projected the build-up and release of chlorine atoms in the stratosphere and concluded that ozone depletion



was likely and would be long-lived, even with remediation. This work has been borne out, in detail, by nearly four decades of research.

Rowland and Molina's work started an environmental movement that began with scientists, led by Rowland, urging the elimination of CFCs. It remains the best success story for global cooperation on a worldwide environmental threat. The activism led to the 1978 ban by the US Environmental Protection Agency on CFC use in aerosol cans, and finally in 1990 to the complete phasing out of CFC production by the Montreal Protocol and its amendments.

In his unwillingness to back down from the implications of his work, Rowland became a role model to many of us, and remains so. This was a threat to some — particularly the CFC industry, but also, less understandably, to some scientific colleagues. For many years, Rowland experienced personal threats as well as irrational attacks on the science.

Rowland's science always stood tall, as did he, and seemed inerrant. He kept up his

interests in ozone and environmental policy, but his research moved on. Soon after the ozone hole was discovered over Antarctica, he made major contributions with his graduate student Neil Harris to the detection of ozone depletion over the Northern Hemisphere. This work was crucial in persuading DuPont and other chemical companies to abandon CFCs in favour of hydrochlorofluorocarbons, which are less damaging to the ozone layer.

In the late 1970s, Rowland initiated a programme to monitor background concentrations of various gases, and that continues today. Six of its group members were working in the field when he passed away. His curiosity demanded an objective approach, and so it was when, working with his former student and then fellow professor (D.R.B.), he identified in 1995 that the unusual mix of high ozone and hydrocarbons in Mexico City was due to leaking propane stoves and heaters, rather than traffic. In 2011, he was involved in discussions regarding the mix of atmospheric hydrocarbons resulting from the Deepwater Horizon oil spill in the Gulf of Mexico.

Over almost five decades, Rowland was active in his research lab as well as teaching, playing tennis and having collegial discussions over lunch. When not travelling, he could be seen carrying his briefcase in one hand, with a pile of papers under the other arm, to and from his office. He was a prolific note-taker, filling a notebook in a week. This practice intimidated one of us (M.J.P.), who, while giving a talk at an international conference, first encountered Sherry in the front row, taking assiduous notes and then asking a terrifying, brutal, yet constructive question.

Rowland treated everyone like a colleague. He disarmingly considered questions from any listener with the depth and profundity due a scientific peer. This trait was appreciated by students, friends and family. To Sherry, the question was of foremost importance; it was at the core of his scientific quest. His passing ended a unique career that merged chemistry and atmospheric sciences, leading to a new partnership between science and policy for the protection of our planet. ■

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NEWS & VIEWS The Titanic disaster, as reported in *Nature* a hundred years ago **p.174**

REVIEW Towards more efficient copper and palladium catalysts **p.177**

LETTER The role of aerosol emissions in climate change **p.228**

PUBLISHED 8 APRIL 2012

CELL BIOLOGY

Centromeres and transcription

Centromere identity is thought to be propagated epigenetically through stable inheritance of nucleosomes that contain the histone H3 variant CENP-A. Rito Gassmann *et al.* now show that in the nematode *Caenorhabditis elegans*, in which centromere activity and CENP-A are distributed along the entire length of the chromosome, embryonic CENP-A domains are established in an inverse pattern to genomic regions that are transcribed in the germline. They propose a model in which germline transcription defines the genomic regions that exclude CENP-A incorporation during embryogenesis. The work links centromere identity to transcription, and could help to explain the evolutionary plasticity of centromeres. **An inverse relationship to germline transcription defines centromeric chromatin in *C. elegans***
R Gassmann *et al.*
doi:10.1038/nature10973

BIOCHEMISTRY

Regulation of glucose production

During fasting, glucose production in the liver is stimulated by an increase in blood glucagon, and the cyclic AMP pathway increases gluconeogenic gene expression through the CREB coactivator CRT2. This study demonstrates interplay between cyclic AMP and calcium signalling pathways,

and the participation of inositol-1,4,5-trisphosphate receptors in regulating CRT2 and hepatic gluconeogenesis under fasting conditions and in diabetes.

Inositol-1,4,5-trisphosphate receptor regulates hepatic gluconeogenesis in fasting and diabetes

Y Wang *et al.*
doi:10.1038/nature10988

GENOMICS

Messenger RNA stability

Secondary RNA structures, or the pairing of complementary sequences within RNA, can regulate the binding of proteins that affect transcript stability, splicing and localization. Such effects are also influenced by the underlying RNA sequence. Hani Goodarzi *et al.* use a computational approach to determine which secondary structures have the greatest impact on RNA behaviour overall. This allows them to develop a map that integrates sequence information, secondary structure, regulatory interactions and targeted pathways.

Systematic discovery of structural elements governing stability of mammalian messenger RNAs
H Goodarzi *et al.*
doi:10.1038/nature11013

PUBLISHED 11 APRIL 2012

EARTH SCIENCE

Core values for iron

The thermal and electrical properties of iron are important for understanding the thermal evolution of the deep Earth and

the power available to drive the dynamo that generates Earth's magnetic field. Previously, these parameters have been estimated by extrapolating results from lower pressure or temperature conditions, but Monica Pozzo and colleagues now present a first-principles calculation of these parameters at the pressure and temperature of Earth's outer core. Both conductivities are found to be two to three times higher than earlier estimates, prompting a re-evaluation of power estimates for the dynamo. The higher values results greatly restrict models for powering the geodynamo and indicate that the top of the core must be thermally stratified.

Thermal and electrical conductivity of iron at Earth's core conditions
M Pozzo *et al.*
doi:10.1038/nature11031

ANIMAL BEHAVIOUR

Bird navigation off course

How do migratory birds detect magnetic fields? Previous studies described a magnetic sensing system consisting of magnetite-containing dendrites in the upper beak, which were thought to support the animals' ability to navigate via magnetic fields. That widely accepted model is now questioned. Christoph Treiber *et al.* show that the iron-rich cells in the beak of the pigeon *Columba livia* are in fact macrophages, not magnetosensitive neurons. Iron-rich macrophages are not unique to the bird's beak. Thus, the neural basis of magnetoreception in birds remains elusive.

Clusters of iron-rich cells in

the upper beak of pigeons are macrophages not magnetosensitive neurons
C D Treiber *et al.*
doi:10.1038/nature11046

GENOMICS

Genome organization

The spatial organization of the genome is linked to biological function, and advances in genomic technologies are allowing the conformation of chromosomes to be assessed across the genome. Two groups present complementary papers on the subject. Elphège Nora *et al.* use chromosome conformation capture carbon-copy (5C) technology and high-resolution microscopy to map the chromosomal interactions over a large region of the mouse X chromosome. They reveal a series of discrete topologically associating domains, as well as a long intergenic RNA that may have a regulatory role. Jesse Dixon *et al.* use an adaptation of the chromosome conformation capture (3C) technique to investigate the three-dimensional organization of the human and mouse genomes in embryonic stem cells and in terminally differentiated cell types. Large, megabase-sized chromatin interaction domains, termed topological domains, are found to be a pervasive feature of genome organization. **Spatial partitioning of the regulatory landscape of the X-inactivation centre**
E P Nora *et al.*
doi:10.1038/nature11049
Topological domains in mammalian genomes identified by analysis of chromatin interactions
J R Dixon *et al.*
doi:10.1038/nature11082

CLIMATE SCIENCE

Aerosols and Atlantic aberrations

A cutting-edge global climate model links atmospheric aerosol emissions to temperature variability in the North Atlantic Ocean, suggesting that human activity influences extreme weather events. [SEE LETTER P.228](#)

AMATO EVAN

Over the past century, the surface of the North Atlantic Ocean has gone through warm and cool periods that are not observed in other ocean basins. This Atlantic Multidecadal Oscillation (AMO)¹ is thought to affect climate processes² ranging from the current high levels of Atlantic hurricane activity to the devastating sub-Saharan droughts of the early 1980s. Although the influence of the AMO on extreme weather events has long been recognized, the physical processes underlying these temperature changes are not understood. On page 228 of this issue, Booth *et al.*³ report their use of a state-of-the-art model of Earth's climate to demonstrate that, at least over the past century, the AMO is largely the response of the upper ocean to changes in the concentration of pollution aerosols in the atmosphere. If correct, their results imply that the influence of human activity on the Atlantic regional climate is more pervasive than previously thought⁴.

The AMO is best depicted as the difference between average ocean surface temperatures over the North Atlantic and those over the global oceans⁵ (Fig. 1). It therefore reflects the deviation of the North Atlantic Ocean from global mean temperatures, which are dominated by the long-term warming that is forced by greenhouse gases. Conventional wisdom has held that the AMO is the natural result of internal processes in the Atlantic Ocean — most notably, fluctuations in deep-ocean circulation, as supported by multi-century climate-model studies⁶.

Booth *et al.*³ simulated the climate of the past 150 years using a version of a well-known climate model⁷ that includes up-to-date parameterizations of aerosol emissions, aerosol chemistry and interactions of aerosols and clouds. Nearly all of the observed decadal variability in North Atlantic surface temperatures was reproduced in their simulation, including

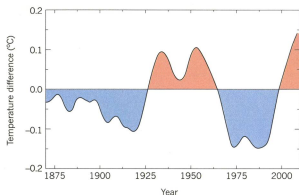


Figure 1 | The Atlantic Multidecadal Oscillation. The difference between average ocean surface temperatures over the North Atlantic and those over the global oceans has oscillated between cool and warm phases over the past hundred years or so, as depicted here. Booth *et al.*³ report that simulations of global climate link this temperature variability to atmospheric aerosol emissions.

the AMO and the long-term warming associated with increasing amounts of greenhouse gases. This is the first time that changes in sea surface temperatures have been reproduced to this degree of accuracy by a climate model. The authors' analysis of the model's output reveals that this variability about the upward temperature trend results from cooling associated with periodic volcanic eruptions, and from the build-up of polluting aerosols in the atmosphere that occurred from pre-industrial times until the late 1960s and early 1970s, when clean-air legislation in the United States and Europe was implemented.

So how do aerosols affect sea surface temperatures? When suspended over water, aerosols tend to cool the surface by increasing the local albedo (the ability to reflect sunlight), a phenomenon known as the aerosol direct effect. Aerosols caused by human activity may also act as nuclei around which water vapour in the atmosphere can condense. When more of these nuclei are available for condensation within a cloud, the number of water droplets in the cloud goes up and the average size of the water droplets goes down, making the cloud brighter so that it reflects more sunlight back out to space. This process is known as the cloud albedo effect, or the first aerosol indirect

effect. It is these aerosol-cloud interactions that have the most influence over the AMO in Booth and colleagues' model.

The idea that variations in aerosol concentration have caused decadal-scale changes in surface temperatures in the North Atlantic is not new. A body of work has emerged suggesting that elements of the AMO are externally forced by aerosols through direct and first indirect effects, and has implicated aerosols from volcanic eruptions⁸, West African dust storms⁹ and human activity¹⁰. However, before Booth and colleagues' work, no study had incorporated the direct and indirect forcings from these various aerosol types to paint a coherent picture of temperature changes in the Atlantic Ocean that was consistent with both the observed

temporal variability and the dominant spatial structure of the changes.

Booth and colleagues' evidence³ that the AMO is caused by changes in the regional abundance of aerosols is compelling, but their results are sensitive to model parameterizations of microphysical processes, particularly the interaction between cloud water droplets and aerosols, that are not well constrained by observations. In addition, their model was unable to reproduce observed multidecadal variability in outbreaks of African dust storms¹⁰, which alter the temperature of the tropical Atlantic¹¹; this may explain why the model does a poorer job of simulating temperatures in the tropical North Atlantic Ocean than it does in the extratropical regions. Furthermore, the authors' conclusion that internal variability of the Atlantic Ocean had a negligible role in shaping the AMO during the twentieth century is at odds with the findings of several previous studies^{6,11}. The reason for this discrepancy is not clear.

If Booth and colleagues' results³ can be corroborated, then they suggest that multidecadal temperature fluctuations of the North Atlantic are dominated by human activity, with natural variability taking a secondary role. This has many implications. Foremost among them is

³This article and the paper under discussion⁷ were published online on 4 April 2012.

that the AMO does not exist, in the sense that the temperature variations concerned are neither intrinsically oscillatory nor purely multidecadal.

Another implication concerns hurricanes. As noted earlier, quiescent and active periods of Atlantic hurricane activity have been linked² to the AMO. These swings in hurricane frequency and intensity might therefore be the regional response to variations in the concentration of pollutant aerosols against a background of global warming, and thus completely man-made. Similarly, human activity might have caused periods of drought within the Sudano-Sahel region of Africa and in northeastern Brazil.

As we try to predict the climate in a warming world, an increasing body of work suggests that aerosols may have regional effects as great as those caused by the global increase in atmospheric carbon dioxide. Booth and colleagues' work³ underscores the importance of understanding the diverse pathways by which humans alter the climate. ■

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GENE EXPRESSION

Running to stand still

Transcription factors regulate the expression of genes by binding to certain DNA sequences. But the outcome can be markedly different, depending on whether the binding is stable or short-lived. SEE LETTER P.251

TOMMY KAPLAN & NIR FRIEDMAN

To reproduce, differentiate or even just respond to changes in their surroundings, cells need to control the expression of thousands of genes. One way of doing this is to use transcription factors — proteins that bind to regulatory regions on their target genes and either activate or repress the transcription of DNA into RNA. Over the past decade, researchers have analysed the binding sites of hundreds of these proteins on the genomes of many organisms and cell types, and measured the gene-expression patterns within the same cells. In such studies, the overall degree of occupancy by a transcription factor at a regulatory region is commonly interpreted as an indication of the protein's ability to control the expression of the gene. However, transcription factors also bind to thousands of genes in a weak, and probably non-functional, manner¹. On page 251 of this issue, Lickwar *et al.*² illuminate this matter by reporting the results of a systematic, genome-wide study of the binding dynamics of a particular transcription factor. The authors find that transcription levels have a stronger link to the kinetics of binding than to the total occupancy of the factor.

The DNA-binding sites of the transcription factor Rap1 along the genome of the yeast *Saccharomyces cerevisiae* were mapped more than a decade ago³. The mapping used a genome-wide protein–DNA binding assay, known as chromatin immunoprecipitation (ChIP)—on-chip, or ChIP-seq, which identifies the genomic locations of a transcription factor over a huge number of live cells and therefore

averages the transcription-factor occupancy over a large population. This is still the method of choice in similar genome-wide studies. However, a high occupancy of a transcription factor at a specific site — as detected by this technique — can mean either that the factor is constantly bound to this DNA location in

some of the cells, or that it is transiently bound in many cells.

To distinguish between the two possibilities, Lickwar *et al.*² adapted a strategy, previously used to measure the turnover of DNA-bound proteins^{4–6}, to address the question of transcription-factor binding stability. The authors created yeast cells that produced two Rap1 variants, Rap1–Flag and Rap1–Myc, each one with a 'tag' consisting of a specific peptide that could be recognized by antibodies. Furthermore, Rap1–Flag was produced constantly, whereas Rap1–Myc's expression was experimentally inducible. The authors then measured the binding of each Rap1 variant to the yeast genome in a dense time series after Rap1–Myc induction. Although the inducible protein quickly outcompeted Rap1–Flag at

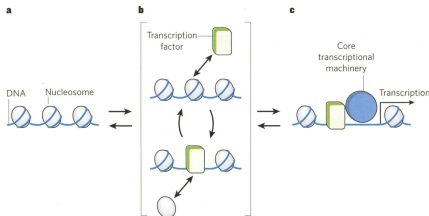


Figure 1 | Well-balanced gene expression. Transcription factors can activate the expression of genes by binding to certain regulatory regions on the genome. Lickwar *et al.*² studied, at the genomic scale, the binding dynamics of one of these proteins, and propose the following model for transcription-factor function. **a**, Most of the genome is wrapped around histone proteins to form nucleosomes, the basic units of DNA packaging into chromatin. **b**, Nucleosomes and transcription factors compete for binding to some regulatory regions. Transcription-factor binding to these regions occurs in short pulses, which are not sufficient for efficient transcription of the gene into RNA. **c**, When the transcription factor binds to its target site for longer periods, it recruits the core transcriptional machinery required to start transcription, leading to high transcription rates.

some genomic sites, it was slowly incorporated into other sites, which suggested that Rap1 binding to the first sites was less stable than to the other sites.

The researchers then applied a mathematical model¹ to estimate the rate of Rap1 turnover at more than 400 target genes. They found that, among genes with high Rap1 occupancy, those with slower Rap1 turnover showed higher transcription levels than those with faster Rap1 turnover. That is, the transcription level depended on how long Rap1 remained bound. Of note, the genomic sites that exhibited fast Rap1 turnover in this analysis¹ have previously been reported^{2,3} to have fast turnover rates of nucleosomes (protein complexes around which DNA is packaged) and of the general transcription factor TBP (which facilitates the binding of the core transcriptional machinery). Overall, the results are consistent with those of other studies^{2,3} that showed that transcription factors and nucleosomes compete for some genomic sites and that this competition leads to inefficient transcription.

Lickwar *et al.* suggest a model for the binding dynamics of transcription factors that activate transcription (Fig. 1). In this model, on binding to a target site, the factor has to recruit the core transcriptional machinery. This process takes some time. Therefore, if the factor's binding to the DNA site is unstable, it will not lead to productive transcription. Indeed, it has been shown⁴ that short, repeated pulses of Msn2 — another transcription factor — into the cell nucleus do not activate target genes, whereas longer pulses do. Therefore, for transcription factors to be effective activators, they require stable binding to their target DNA.

Moreover, the researchers speculate that a constant turnover or 'treadmilling' of nucleosomes and transcription factors acts as a distinct mechanism for transcriptional regulation. Unlike static gene repression¹⁰, in which transcription is prevented by the nucleosome's protection of DNA, a site that has a treadmill transcription factor is poised for activation. When, somehow, the nucleosome is removed or its affinity for DNA is decreased, the factor can quickly achieve stable binding to its target sequence and so activate the gene's transcription. Several mechanisms would allow for the targeted eviction of nucleosomes, including chromatin-remodelling enzymes (which move nucleosomes on DNA), chemical modifications of histones (the protein components of nucleosomes) or the replacement of certain histones with specific variants.

Lickwar and colleagues' study¹ explains how different regulatory regions can present similar levels of transcription-factor occupancy and different transcriptional levels. But it also raises further exciting questions. Do the different turnover rates of transcription factors play a key part in gene regulation? Or do they just reflect some other aspects of the transcriptional

process, such as stabilization of protein–DNA binding by interactions with the transcriptional machinery? Are nucleosomes the only competition for factor binding to DNA, or is competition with other transcription factors important too?

To fully understand how transcription factors work, we should consider not only their overall binding occupancy, but also their binding dynamics. This line of research will form the basis for a much-needed quantitative understanding of transcription regulation kinetics. ■

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ASTROPHYSICS

Fresh light on stardust

Ageing stars produce elements vital for life and disperse them into space on stellar winds. The discovery of large dust grains in the vicinity of cool giant stars sheds light on the mechanisms that drive such winds. SEE LETTER P.220

SUSANNE HÖFNER

Chemical elements that are crucial for building Earth-like planets and living organisms have their origin in ageing stars and stellar deaths. The nuclear processes that create these elements are well understood, but the mechanisms that transport them to the stellar surfaces and out into interstellar space are still a matter of intensive research. On page 220 of this issue, Norris *et al.*¹ report the detection of silicate particles about 600 nanometres in diameter in the immediate vicinity of several cool giant stars. This result confirms the predictions of models² that explain how gas can escape stellar gravity and become part of the cosmic-matter cycle.

Stellar explosions known as supernovae have considerable input into the production and dispersion of heavy elements (those heavier than helium), but they are not the sole contributors. Stars, including the Sun, release continuous outflows of gas, called stellar winds, for most of their lives. As stars evolve into cool giants and supergiants, stellar winds become increasingly effective in transporting matter out of stellar-gravity wells, enriching the surrounding interstellar medium with newly produced chemical elements. Winds of ageing low- and intermediate-mass stars, such as those observed by Norris *et al.*¹, lead to a runaway mass-loss process that eventually

stops the stars' nuclear processes and turns them into white dwarfs.

The mechanisms that drive gas away from stars differ, depending on a star's surface temperature, mass, luminosity, chemical composition and magnetic field. Winds of cool, luminous giants are presumably triggered by radiative acceleration of dust grains that form in the extended stellar atmospheres. Momentum is transferred from the photons emitted by the star to the dust grains through photon absorption and scattering, and is subsequently redistributed to the more numerous gas particles by collisions with the dust grains (Fig. 1). Because the star's photons are predominantly directed away from the stellar surface, the flow of gas and dust also follows this pattern.

Although the physical principles of dust-driven winds are reasonably well understood, there is currently no consensus on which types of grain are driving the outflows. However, some basic features are known. First, the mass of the gas that is pushed outwards by the dust is more than 100 times higher than the collective mass of the dust particles. This requires grains made from abundant materials that have large radiative cross sections to drive the winds. Second, the grains have to form close to the star to trigger the outflows. This distance is limited by how far shock waves, caused by pulsation and convection in the stellar interiors, can lift gas above the stellar surface, at

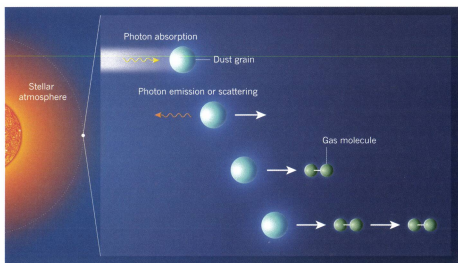


Figure 1 | Dust-driven stellar winds. Dust grains forming in the atmosphere of a cool luminous star are accelerated away (white arrow) from the star through absorption and emission or scattering of stellar photons. By subsequently colliding with molecules in the surrounding gas, the grains accelerate the molecules, make them collide with other gas molecules and trigger an outflow of gas, or stellar wind. Norris and colleagues' study¹ of the immediate vicinity of several cool giant stars provides information on the sizes and material properties of the grains that drive stellar winds.

which temperatures are typically 3,000 kelvin, too high for solid particles to form. The strong flux of stellar photons — which not only accelerates but also heats the grains — severely limits the types of dust material that can form and survive at distances at which winds are triggered.

On the basis of element abundances and distinctive spectral features, magnesium iron silicates of the olivine and pyroxene type have long been considered to be promising candidates for wind drivers². But detailed models demonstrate⁴ that silicate grains have to be almost devoid of iron to exist at the distance from the stars at which the winds are accelerated, in contrast to earlier assumptions⁵. Because such particles are highly transparent at near-infrared wavelengths, at which most of the stellar flux is emitted, photon absorption will be insufficient to trigger an outflow. If, however, such iron-free silicate grains grow to 100–1,000 nm in radius, photon scattering dominates over absorption, providing sufficient radiative acceleration for driving winds⁶.

It is therefore of note that silicate grains collected in the Solar System by NASA's Stardust mission⁷ tended to be iron-poor. However, whether this reflects the original composition of grains produced in previous generations of stars before the Solar System formed, or whether stardust is heavily modified in interstellar space and during planetary-system formation, is unclear. To test theoretical predictions, the size and composition of dust grains have to be measured in the immediate vicinities of the stars in which they are formed.

By analysing starlight scattered on dust grains close to several cool giants, Norris

*et al.*¹ found silicate particles with diameters of 600 nm at distances of about two stellar radii, in accordance with model predictions². This result was made possible by a clever combination of advanced instrumentation and observational methods. The authors used measurements of polarization (the direction in which light's electric field oscillates) to identify photons scattered by dust and to determine the size of the grains, and employed interferometric

MICROBIOLOGY

Adaptation by target remodelling

Bacteria direct their movement in response to certain chemicals by controlling the rotation of whip-like appendages called flagella. The sensitivity of the response can be adjusted at the signal's target, the flagellar motor. SEE LETTER P233

GERALD L. HAZELBAUER

When we switch on a lamp in a dark room, our eyes adapt quickly to the lighting. Similarly, in a process known as chemotaxis, specific sensory systems in bacteria detect and adapt to changes in the concentration of molecules such as nutrients, so that the microbes can control the motion of their flagella and swim to more favourable environments. Many adaptive mechanisms act on cell-surface receptor proteins that detect stimuli and generate signals across the cell membrane and within the cytoplasm. Indeed,

techniques to obtain high-spatial-resolution images of the close stellar environments in which the winds are triggered.

Identifying wind-driving dust grains in cool giants is a step towards a comprehensive picture of stellar-mass loss, which is essential for understanding ageing stars and their role in the cosmic-matter cycle. Stellar winds strongly affect the evolution of low- and intermediate-mass stars. These stars are probably the progenitors of type Ia supernovae, which are a cornerstone of cosmological studies^{8,9}. Reliable mass-loss rates and dust yields are essential ingredients for understanding the chemical evolution of the Milky Way and other galaxies, and for estimating the effects of interstellar dust on the light of distant galaxies, which is used in studies of the early Universe. The advanced technique used by Norris *et al.*¹ for studying the properties of stardust at its place of origin provides crucial input to these fields of research. ■

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the sensitivity of the bacterial chemotaxis machinery is regulated by addition or removal of methyl groups on the chemical-sensing receptors (chemoreceptors)¹. But, as Yuan *et al.*² report on page 233 of this issue, the machinery can also adapt by remodelling the target of signalling — the flagellar motor. Motor adaptation differs from receptor adaptation in its time frame, mechanism and function.

In the bacterial chemotaxis system, different chemoreceptors can bind to either attractant or repellent ligand molecules, and by doing so they alter the activity of a receptor-associated

NATURE

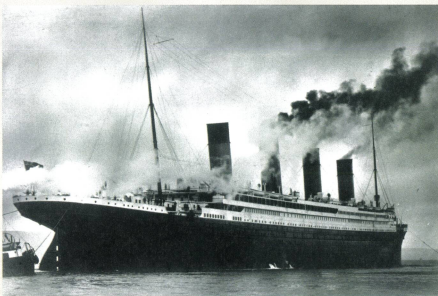
100 Years Ago

The loss of the "Titanic"

The terrible loss of life on account of the disaster to the *Titanic* has directed emphatic attention to various aspects of the employment of wireless telegraphy in times of crisis at sea. The point which is at the moment attracting most of the public attention is that of the erroneous messages, or alleged messages, which appeared in the newspapers in the day or two following the disaster ...

All this raises more prominently than ever the chaotic condition of wireless telegraphy in the United States ... [T]he most urgent call for help will pass unheeded if none of the operators on the ships within hail are on duty. In fact, it seems to have been a mere chance that the *Carpathia* operator was at his apparatus at the time the *Titanic* called. On ships that carry only one operator — and very few carry more — the man cannot always be on the look-out ...

Engineering aspects of the disaster are discussed in the leading article in *Engineering* for April 19 ... [S]everal questions present themselves as ripe for discussion and settlement. The effect of centre-line or longitudinal wing bulkheads is one of these. Such have advantages in confining any water admitted to a part of the width, but have disadvantages even from the point of view of stability under disastrous conditions. The effect of impact on the superstructure of very large ships will have to be considered. In such ships it has become a practice to have two or three decks above the moulded structure. Would inertia have effects somewhat similar to those experienced in railway collisions, in which the body of the carriage is driven from the



Titanic sank on 15 April 1912 on its maiden voyage, after hitting an iceberg off Newfoundland.

under-frame? ... The engineers of the ship have all been lost — their claim to recognition is the simplest and best; they did their duty to the end.

From *Nature* 25 April 1912

At the time when the *Titanic* was lost the standing Advisory Committee appointed by the Board of Trade under the provisions of Merchant Shipping Acts was engaged in the reconsideration of the regulations for boats and life-saving appliances ... The main recommendations of [their] report may be summarised. First, it is recognised that "the stability and seaworthy qualities of the vessel itself" must be regarded as of primary importance. This includes the question of watertight subdivision, now under investigation by a special committee. Second, as regards boats and life-saving appliances it is recommended that accommodation should be provided for the total number of persons which each *foreign-going* passenger steamship is licensed to carry ... One of [the committee's] most valuable recommendations is that proposing to extend the present regulations and to prescribe to those in charge of ships the necessity for

proceeding at moderate speed "at night in the known vicinity of ice."

From *Nature* 29 August 1912

It behoves surely men of science to ask the question whether we have not reached the imperative limits of that false security which the "practical man" is wont to feel in his contempt for scientific "theory"; and further, whether the time has not therefore come for legislation requiring commanders of the largest ocean-going steamers to hold a diploma, guaranteeing such a systematic course of study (say in a class at Greenwich or Kensington) in marine physiography and the elementary laws of mechanics as would quicken their imagination as to the uncertainty and the magnitude of the risks to be run in an abnormally ice-drifted sea. Lord Mersey's report may whitewash the facts, but the facts *en évidence* remain; and the chain of cause and effect in the lamentable and tragic loss of the *Titanic* leads us in the last resort to the notorious contempt for scientific acquaintance with the facts and laws of nature on the part of the "practical man".

From *Nature* 12 September 1912

kinase enzyme² (Fig. 1). The kinase adds a phosphate group to the protein CheY, which can bind only in its phosphorylated form (CheY-P) to FliM, a protein component of the flagellar rotary motor. Binding leads to a switch in flagellar rotation from anticlockwise — the default state — to clockwise. As anticlockwise rotation powers forward swimming ('runs') and clockwise rotation causes abrupt directional changes ('tumbles'), their alternation generates a three-dimensional random walk. When the bacterium swims towards increasing

concentrations of attractant, the interaction of attractant with its receptors reduces kinase activity, and thus CheY-P levels and the probability of tumbles, biasing the random walk in favourable directions.

But when ligand concentration remains constant, the system adapts by reverting to its null state, so that it can then respond to any additional concentration changes. Adaptation occurs through the addition or removal of methyl groups to the receptors in response to occupancy by attractants or repellents.

Methylation and demethylation shift receptor conformation and thus kinase activity, CheY-P levels, rotational bias and swimming behaviour in the opposite direction to attractant and repellent occupancy, respectively. These receptor adaptive changes occur more slowly than those generated by ligand binding, and this difference provides the bacterium with a means of comparing current and recent ligand concentrations through the opposing influences of receptor occupancy and the extent of methylation. The overall result is

that the microbe can sense temporal changes in the concentration of relevant molecules and therefore can swim in the 'right' direction.

The flagellar motor is extremely sensitive to small variations in CheY-P concentration³, and this poses a challenge to the system because the steady-state CheY-P concentration varies among individual cells⁴. Given this variation, some cells would be expected to run only or to tumble only, and therefore to be unable to perform proper chemotaxis. As this does not seem to occur, a mechanism must exist to keep a correspondence between steady-state CheY-P levels and the narrow CheY-P concentration range over which the rotational switch is sensitive. However, previous research⁵ did not find one possible mechanism — feedback from the flagellar rotary motor to the kinase.

Yuan *et al.*² investigated an alternative possibility: could the motor itself adapt to changes in CheY-P concentration? The authors used a strain of *Escherichia coli* that lacked the enzymes that carry out receptor methylation and demethylation, as otherwise the process under study would have been masked by the rapid and complete adaptation mediated by these enzymes. When the bacteria were stimulated by an attractant, the authors observed that the CheY-P concentration and the motor's clockwise bias both decreased rapidly, as expected. However, over the next few minutes, even though CheY-P levels remained unchanged, the clockwise bias gradually increased to an intermediate steady-state value that was lower than in the absence of attractant. A similar slow, partial adaptation of the flagellar motor was reported⁶ 25 years ago in bacteria lacking the receptor methylation and demethylation enzymes.

The researchers² also measured the clockwise bias of flagellar motors in individual bacterial cells before stimulation, at maximum response and after partial adaptation. In the presence of persistently decreased CheY-P concentration, they documented a shift in the motor's sensitivity to CheY-P to a lower, but still narrow, concentration range. When they analysed these data using a mathematical model for cooperative motor switching, the results indicated that the adaptation probably reflected an increased number of binding sites for CheY-P in the flagellar motor — in other words, more copies of FlIM.

To test this hypothesis, the authors expressed a modified version of FlIM (fused to a yellow fluorescent protein) in the *E. coli* cells, and then observed these cells using total internal reflection fluorescence (TIRF) microscopy. The researchers did indeed detect increases of up to about 25% in the number of FlIM copies (from 34 to 42 copies) in flagellar motors upon partial adaptation. This result is consistent with a previous finding⁷ of CheY-P-dependent turnover of FlIM units, and suggests a function for such a process: the addition of FlIM copies to the flagellar motor would increase the

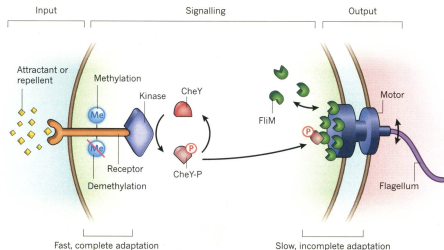


Figure 1 | Microbial sense and sensibility. Protein receptors on a bacterial cell's surface bind to specific attractant or repellent molecules, which act as inputs for the bacterium's sensory system. In the absence of attractant, a receptor-associated kinase enzyme donates a phosphoryl group (P) to the protein CheY. Phosphorylated CheY (CheY-P) is an intracellular signal that interacts with the protein FlIM at the flagellar motor. This interaction generates a change in rotational direction of the motor — the system's output. Upon attractant binding, the receptor undergoes a conformational change that reduces kinase activity and consequently the levels of CheY-P and the probability of a change in the direction of rotation. Binding of repellent has the opposite effects. If attractant or repellent concentrations do not change for a while, the sensory system 'resets' rapidly and completely, within seconds, to a null state through receptor methylation (for attractant) or demethylation (for repellent) at the input end. Yuan *et al.*² find that the system also slowly and partially adapts at the output end to different, persistent levels of CheY-P. It does so by changing the number of FlIM copies in the flagellar motor, thus shifting the narrow sensitivity range of the motor to correspond to different steady-state levels of CheY-P.

probability of CheY-P binding to the motor and therefore the sensitivity of the rotational switch.

Yuan and colleagues' results² generate questions for future research. For instance, how does the flagellar motor sense changes in the level of CheY-P? And how does sensing lead to an increased number of FlIM copies? How few and how many copies can be accommodated, and what structural interactions between FlIM units allow such variability? Answering these questions will be challenging, but insight could be gained by identifying and characterizing mutant bacteria that are defective in motor adaptation, and by extending high-resolution fluorescence analyses such as the TIRF microscopy experiments done by the authors. Full answers are likely to require both biochemical and structural studies.

It also needs emphasizing that motor adaptation is a slow process, occurring over minutes. So, in the absence of methylation-dependent adaptation of the receptors, which occurs within seconds, bacteria cannot perform chemotaxis^{8,10}. Nonetheless, motor adaptation is likely to be crucial for accommodating stochastic variations in the dosages of protein components of the chemotaxis sensory machinery, such as CheY. The adjustment of the sensitivity of a signalling system by adaptive remodelling of its output end is a

tantalizing observation, because experience tells us that if a new strategy is discovered in one biological system, then there are undiscovered examples in other systems. Therefore, as the authors suggest, other biological molecular machines may adapt to changes in signal levels by resetting the sensitivity of their response. ■

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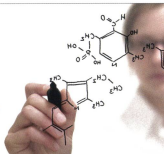
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High-valent organometallic copper and palladium in catalysis

Amanda J. Hickman¹ & Melanie S. Sanford¹

Copper and palladium catalysts are critically important in numerous commercial chemical processes. Improvements in the activity, selectivity and scope of these catalysts could drastically reduce the environmental impact, and increase the sustainability, of chemical reactions. One rapidly developing strategy for achieving these goals is to use 'high-valent' organometallic copper and palladium intermediates in catalysis. Here we describe recent advances involving both the fundamental chemistry and the applications of these high-valent metal complexes in numerous synthetically useful catalytic transformations.

Homogeneous copper- and palladium-catalysed reactions are widely used for the construction of important organic molecules, including pharmaceuticals^{1–3}, commodity chemicals⁴ and polymers⁵. The development of copper and palladium catalysis has been inextricably linked because both metals have been used extensively in the construction of similar types of carbon–carbon and carbon–heteroatom bonds. Furthermore, advancements in, and insights into, copper chemistry have often spurred improvements in palladium-catalysed processes, and vice versa, leading to a wide range of robust, synthetically valuable and often complementary synthetic methods. This Review focuses particularly on an area that has progressed tremendously over the past decade: the use of high-valent copper and palladium complexes in catalysis (see Box 1 for definitions of 'high-valent' copper and palladium).

History and importance of copper catalysis

Copper is an inexpensive, earth-abundant, non-toxic metal that has found widespread application in homogeneous catalysis. For example, copper-catalysed cross-coupling reactions have been extensively studied since their discovery at the turn of the twentieth century^{6,7}. Such reactions serve as versatile methods for synthesizing biaryl linkages as well as for constructing the carbon–heteroatom bonds of aryl amines, aryl ethers and aryl thioether derivatives. The alkylation of carbon electrophiles with organometallic copper compounds (organocuprates) is another classic reaction in organic synthesis⁸. Diverse carbon–carbon bond-forming reactions of organocuprates have been developed over the past 70 years, and these transformations are featured in most introductory undergraduate textbooks on organic chemistry.

High-valent copper intermediates (that is, organometallic copper(III) (Cu(III)) species) have long been proposed to have a role in both copper-catalysed cross-coupling and organocuprate reactions⁹. In particular, carbon–carbon and/or carbon–heteroatom bond formation from an organo-Cu(III) species has been invoked as the product-releasing step of many of these transformations^{8,9,11,12}. However, the proposed high-valent copper intermediates have eluded detection for decades, and as a result there has been considerable controversy over the mechanistic details of both organocuprate additions and copper-catalysed cross-coupling¹¹. Indeed, until very recently, these two transformations were among the least mechanistically understood synthetic methods in organometallic chemistry. These mechanistic questions and controversy have provided tremendous motivation for probing the accessibility and reactivity of organo-Cu(III) species. A deeper mechanistic understanding

of their chemistry promises to allow the development of improved copper catalysts for known reactions as well as to inspire novel copper-catalysed transformations. In the section on high-valent copper below, we discuss recent progress on many of the vital questions in this area (discussed in detail in Box 1).

History and importance of palladium catalysis

Although copper-mediated cross-coupling methods were the first of their kind, today cross-coupling has become synonymous with a different metal: palladium. Well-defined palladium-catalysed cross-coupling reactions were first developed in the 1970s, and they quickly surpassed copper-based methods in both popularity and scope. These reactions have transformed the way organic chemists approach the construction of bonds in complex molecules^{1,5,13}, and the wide-ranging impact of this methodology was recognized in the awarding of the Nobel Prize in Chemistry in 2010.

The rapid success of palladium-catalysed cross-coupling is due, in large part, to extensive and systematic investigations of reaction mechanisms. Mechanistic analysis has revealed that nearly all of these processes involve catalysis by 'low-valent' palladium (that is, palladium in the 0 (Pd(0)) or +2 (Pd(II)) oxidation states). For many reactions, Pd(0) and Pd(II) catalytic intermediates have been well characterized and the steric and electronic influence of supporting ligands on catalysis has been studied in detail¹⁴. Such mechanistic studies have been crucial in the development of new catalyst structures and novel transformations with wide scope and mild reaction conditions^{1,13,14}.

Although catalysis by low-valent palladium is ubiquitous and extremely synthetically useful, it has several important limitations that stem from the fundamental properties of organo-Pd(II) complexes. These include limited reactivity to forming certain important types of chemical bond (for example carbon–halogen and carbon–CF₃ linkages) and a high susceptibility to decomposition pathways such as β -hydride elimination. These challenges have motivated the study of catalysis by high-valent palladium as a potentially complementary mechanistic pathway. The first 30 years of palladium chemistry were dominated by the use of low-valent palladium, but over the past decade the unique reactivity of Pd(III) and Pd(IV) intermediates has increasingly been recognized and exploited in catalysis. In the section on high-valent palladium below, we present recent advances in the field that demonstrate the relevance and utility of high-valent palladium in diverse catalytic applications (discussed in detail in Box 1).

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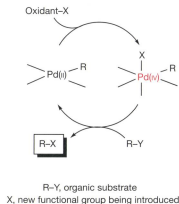
BOX 1

High-valent copper and palladium

In the context of palladium and copper, high-valent compounds include palladium in the +3 or +4 oxidation state (Pd(III) and Pd(IV), respectively) and copper in the +3 oxidation state (Cu(III)). Organometallic complexes of these high-valent metals are defined as molecules that contain copper–carbon or palladium–carbon bonds. Catalysis by high-valent copper or palladium is defined as a catalytic reaction in which the metal is oxidized to form a high-valent organometallic intermediate during the catalytic cycle.

Advances in the chemistry of high-valent copper have enabled researchers to address a number of critical issues, including the synthetic accessibility of organo-Cu(III) complexes, the viability of carbon–carbon and carbon–heteroatom bond formation from discrete organo-Cu(II) species, the catalytic relevance of Cu(II) complexes and the ability to exploit high-valent copper intermediates to improve catalytic reactions and/or discover new reactivity modes.

There has been a renaissance in the chemistry of high-valent palladium over the past decade that has provided key insights into the following issues: the synthetic accessibility of high-valent Pd(III) or Pd(IV) organometallic complexes, the ability of these species to participate in stoichiometric carbon–carbon and carbon–heteroatom bond-forming reactions, the catalytic relevance of these high-valent palladium species and the advantages of high-valent palladium catalysis (in terms of enhanced substrate range, milder reaction conditions and improved chemoselectivity, regioselectivity and/or site selectivity) over more-common low-valent analogues. A representative high-valent palladium catalytic cycle is shown in the figure.



High-valent copper

Before 2000, high-valent organometallic copper complexes were rare. The occasional examples reported in the literature were stabilized by rigid, chelating and/or perfluorinated ligands^{15–17}, as exemplified by compounds 1–3 in Fig. 1a. Although complexes 1–3 are structurally interesting, they do not have the characteristic reactivity that has been attributed to Cu(III) in catalysis. Specifically, they are all inert to carbon–carbon and carbon–heteroatom bond-forming reactions. As such, these compounds were largely considered curiosities, whose relevance to copper catalysis was tenuous at best. The past ten years have seen tremendous developments in this area with the observation and detailed investigation of catalytically relevant organo-Cu(III) species in both carbon–carbon and carbon–heteroatom bond formation. In this section, we will specifically focus on two representative areas: high-valent copper intermediates in carbon–carbon bond-forming reactions of organocuprates and high-valent copper intermediates in carbon–nitrogen and carbon–oxygen couplings.

An early advance in the chemistry of high-valent copper came from investigations of carbon–carbon bond-forming reactions of organocuprates with enones, alkyl halides and allylic electrophiles. Computational studies

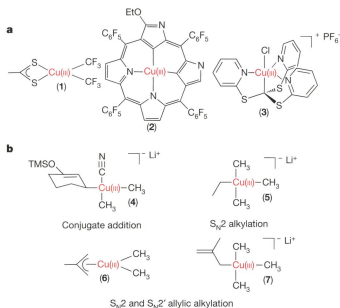


Figure 1 | High-valent copper complexes. **a**, Early examples of isolatable organometallic Cu(III) complexes. **b**, Cu(III) intermediates of organocuprate reactions detected at -100°C using rapid-injection NMR spectroscopy. Et, ethyl; TMS, trimethylsilyl.

of these transformations implicated a Cu(III) intermediate in the key carbon–carbon bond-forming step^{18–20}. However, for many years little experimental evidence was available to support this hypothesis, as the putative Cu(III) compounds proved too transient for detection using standard spectroscopic techniques²¹. In 2007, rapid-injection NMR spectroscopy (RI-NMR) was introduced as a method to directly observe Cu(III) species such as 4–7 in real time (Fig. 1b). Remarkably, when generated using RI-NMR, the Cu(III) adducts could be detected and fully characterized at -100°C (refs 22–26). Furthermore, on being warmed, these discrete organo-Cu(III) intermediates underwent carbon–carbon bond-forming reductive elimination²².

This field is still in its infancy, but the ability to study directly the reactivity of high-valent organo-Cu(III) species has begun to provide mechanistic insights of direct relevance to copper catalysis. For example, Lewis basic additives such as cyanide, phosphines, pyridines and amines have been known for decades to improve the yield and/or rate of organocuprate conjugate addition reactions^{27,28}. Some researchers have proposed that the primary role of these additives is to enhance the solubility of copper starting materials or intermediates²⁸. By contrast, other groups have speculated that these Lewis bases have a more intimate role in the reaction mechanism by binding to copper and tuning its reactivity towards oxidative addition and/or carbon–carbon bond-forming reductive elimination^{19,27}.

RI-NMR has provided a way of interrogating these possibilities directly. A series of Cu(III) complexes of general structure $(\text{CH}_3\text{CH}_2)(\text{CH}_3)_2\text{Cu(III)LB}$, where LB denotes a Lewis basic ligand, were prepared using this technique and evaluated as models for conjugate addition intermediates²⁹. The nature of LB was found to have a profound influence on the stability of these species. For instance, when LB was pyridine, the Cu(III) complex **8** was a short-lived intermediate at -100°C (0.5 h to maximum concentration) (Fig. 2). At this temperature, **8** underwent facile ligand exchange to form the Cu(III) complex $(\text{CH}_3\text{CH}_2)(\text{CH}_3)_2\text{Cu(III)Li}$, as well as carbon–carbon bond-forming reductive elimination to release propane and $(\text{CH}_3)_2\text{Cu(I)}_2\text{Li}$. By contrast, under otherwise analogous conditions the dimethylaminopyridine complex **9** was very stable at -100°C (Fig. 2). Minimal (<10%) reductive elimination was detected, and ligand exchange to form $(\text{CH}_3\text{CH}_2)(\text{CH}_3)_2\text{Cu(III)Li}$ was not observed in this system.

Reference 29 clearly shows that Lewis basic ligands drastically influence the relative and absolute rates of carbon–carbon coupling at Cu(III)

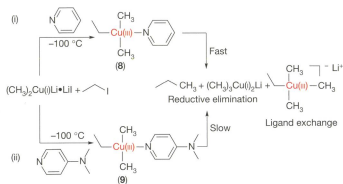


Figure 2 | RI-NMR studies of the effect of Lewis bases on the reactivity of Cu(III) complexes **8** and **9**. Path (i): pyridine-containing intermediate **8** is short lived and undergoes ligand exchange to form $(\text{CH}_3)_3\text{Cu}(\text{II})(\text{CH}_3)_2\text{Cu}(\text{III})\text{Li}^+$ as well as carbon–carbon bond-forming reductive elimination. Path (ii): by contrast, dimethylaminopyridine-containing intermediate **9** is stable at -100°C under analogous conditions.

centres. In the future, more-quantitative RI-NMR analysis of reaction kinetics and ligand electronic/steric effects should provide further insights about the rate- and selectivity-determining steps of conjugate addition and other organocuprate reactions. Such studies will also be invaluable in establishing the role of Cu(III) in the reactions of organocuprates with other electrophiles (for example acyl halides, carbonyl compounds and cyclopropanes). Furthermore, they will provide a mechanistic platform for rationally designing new synthetic methods in this area.

The ability to use RI-NMR to observe and study organo-Cu(III) intermediates also has important implications for emerging areas of copper catalysis. For example, the copper-catalysed carbon–hydrogen arylation of anilides with diphenyliodonium trifluoromethanesulphonate, $[\text{Ph}_2\text{I}]\text{OTf}$, was recently reported³⁰. In these reactions, carbon–carbon bond formation occurs *meta* to the amide substituent, site selectivity that is complementary to palladium-catalysed, ligand-directed carbon–hydrogen arylation methods³¹. The mechanism of this reaction remains controversial. Calculations using density functional theory have implicated the intermediacy of a Cu(III)–phenyl complex³²; however, the accessibility of such intermediates has yet to be confirmed experimentally. Alternative mechanisms such as Lewis acid catalysis are also plausible. RI-NMR would serve as a powerful technique for detecting Cu(III) (if present) during catalysis and/or for interrogating stoichiometric reactions of Cu(I) model complexes with $[\text{Ph}_2\text{I}]\text{OTf}$. Such studies could help to clarify the mechanism of this novel transformation as well as to probe the origin of the *meta* selectivity.

A second key advance in catalysis by high-valent copper came in the study of carbon–heteroatom bond formation from Cu(III) intermediates. As representative examples, we highlight recent investigations of the copper-catalysed amination of aryl bromides and of copper-catalysed carbon–hydrogen bond amination and oxygenation. Copper catalysts are well known to promote the amination of aryl boronic acids^{33,34}, aryl halides^{35,36} and carbon–hydrogen bonds^{37–42}. Common nitrogen heterocycles such as pyrazole, pyridone and phthalimide are particularly effective coupling partners, and the reactions often proceed under mild conditions. Many researchers have proposed the intermediacy of Cu(III) in these transformations^{18,43,44}. However, this hypothesis has been the subject of significant debate, and others have argued that single-electron transfer, halide atom transfer or σ -bond metathesis mechanisms at low-valent Cu(I) or Cu(II) are more likely^{45,46}. Until very recently, no Cu(III) catalytic intermediates had been detected, and carbon–nitrogen bond formation from a Cu(III) complex had not been observed directly.

The synthesis of the first isolatable Cu(III)–monoaryl species (**10**; Fig. 3a), in 2002, was a turning point for this field⁴⁷. Like many of the early examples of organometallic Cu(III) compounds (for example **2** and **3** in Fig. 1), complex **10** is stabilized by an electron-donating macrocyclic ligand. However, unlike its predecessors **10** is remarkably reactive to

carbon–nitrogen bond formation. For example, **10** reacts stoichiometrically with amines such as pyridone, oxazolidinone and acetanilide to form aminated products⁴⁸ (**11**; Fig. 3a). Further study showed that less basic amines reacted faster, implicating deprotonation of the amine at or before the rate-determining step of this sequence. Notably, in the absence of amine, **10** was shown to undergo carbon–bromine bond-forming reductive elimination to release aryl bromide **12** when trifluoromethanesulphonic acid is present (Fig. 3b).

The next key question was whether Cu(III) complex **10** and analogues thereof are relevant to catalytic carbon–nitrogen coupling reactions. The copper-catalysed amination of aryl bromide **12** with pyridone was studied to test this possibility⁴⁹. Remarkably, it was possible to detect a steady-state concentration of Cu(III) intermediate **13** during catalysis by means of ultraviolet–visible and NMR spectroscopy (Fig. 3b). The observation of **13** is consistent with its participation in the turnover-limiting step of the catalytic reaction. Although studies of this one system do not resolve the controversy surrounding the mechanism of all copper-catalysed cross-coupling reactions, they provide the first definitive demonstration that Cu(III) can be catalytically relevant in these transformations.

Pioneering studies have also recently established a role for high-valent copper in certain copper-catalysed carbon–hydrogen bond-functionalization reactions^{37,40}. Before these studies, there was considerable mechanistic uncertainty about these transformations^{38,42,49–53}. Radical pathways initiated by single-electron transfer from amine, enolate and electron-rich arene substrates have frequently been proposed^{42,52}. However, a growing number of examples have been reported with substrates (for example alkynes^{50,51} and electron-deficient arenes^{50,42,52,53}) that are unlikely to participate in such a mechanism. Very recently, the catalytic aerobic carbon–hydrogen oxygenation of macrocycle **14** with methanol (Fig. 3c) was demonstrated⁵⁷. *In situ* ultraviolet–visible spectroscopic studies revealed the build-up and subsequent decay of Cu(III) complex **13** during the catalytic reaction, implicating this species as a catalytically relevant intermediate. Further kinetic studies suggested that the rates of Cu(II)-mediated carbon–hydrogen cleavage and of carbon–oxygen bond formation from Cu(III) are closely matched, which would explain the observed concentration profile of intermediate **13** during catalysis⁵⁷.

In summary, ten years ago little was known about the stability and reactivity of high-valent copper complexes. Since then there has been considerable progress, including the first observation and study of carbon–carbon and carbon–heteroatom bond formation from discrete organo-Cu(III) species in stoichiometric and catalytic transformations. Fundamental studies of organo-Cu(III) are beginning to provide greater understanding of mechanism, which in turn should allow the rational development of new synthetic methods⁵⁴.

High-valent palladium

Sporadic reports over the past 50 years have proposed the intermediacy of Pd(IV) in catalysis^{55–57}. However, these proposals were frequently viewed with scepticism owing to a lack of evidence supporting the viability of such species. Thus, a first key challenge was to determine whether it was possible to form, detect and isolate Pd(III) and/or Pd(IV) complexes from the reactions of Pd(II) precursors with oxidants. Early work established the viability of this approach and demonstrated that electron-donating, rigid, multidentate supporting ligands can be used to stabilize high-valent palladium products. For example, in 1988 organometallic Pd(IV) complex **16** (Fig. 4) was prepared through the reaction of **15** (containing the rigid, bidentate 2,2′-bipyridine ligand) with CH_3I (ref. 58). Similarly, organo-Pd(III) dimer **18** was produced by reacting **17** (containing bidentate, electron-donating cyclometalated phosphines) with PhICl_2 (ref. 59). These seminal discoveries have inspired extensive efforts to exploit related intermediates in catalysis. In this section, we specifically focus on progress in two areas: high-valent palladium intermediates in carbon–halogen bond formation and high-valent palladium intermediates in trifluoromethylation reactions.

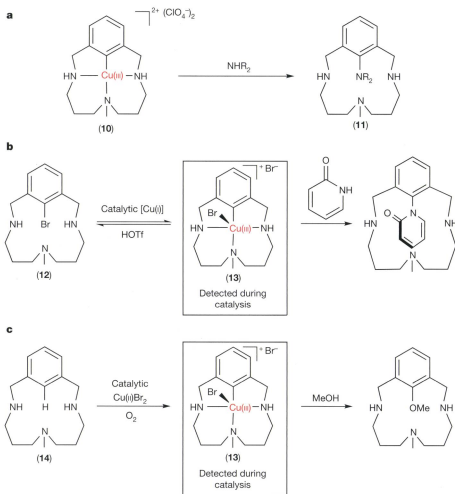


Figure 3 | High-valent copper complexes involved in carbon-heteroatom bond formation. **a**, Stoichiometric carbon-nitrogen bond formation from an isolated organo-Cu(III). NHR_2 represents pyridone, oxazolidinone or acetanilide. **b**, *In situ* observation of an organo-Cu(III) intermediate in the

coupling of aryl bromide **12** with pyridone. Tf, trifluoromethanesulphonate. **c**, *In situ* observation of an organo-Cu(III) intermediate in the oxygenation of carbon-hydrogen bonds. Me, methyl.

The formation of carbon-halogen bonds has been an important target reaction for catalysis by high-valent palladium. Halogenated molecules are valuable starting materials for many organic transformations including nucleophilic substitutions, metal-catalysed cross-couplings and Friedel-Crafts alkylations. Notably, carbon-halogen bond-forming reductive elimination is both thermodynamically unfavourable and kinetically slow from most Pd(II) complexes⁶⁰. As a result, most Pd(II)- or Pd(0)-catalysed transformations of aryl or alkyl halides involve breaking carbon-halogen bonds rather than forming them⁶¹ (Fig. 5a). By marked contrast, recent work has shown that many high-valent palladium complexes promote the facile formation of carbon-halogen bonds^{62–64}. Initial studies in this area focused on generating high-valent organometallic palladium halide complexes through the stoichiometric two-electron oxidation of Pd(II) precursors with electrophilic halogenating reagents^{65–67} (for example Cl_2 , PhICl_2 , N -chlorosuccinimide and XeF_2 (collectively abbreviated X^+ in Fig. 5a)). Depending on the structure of the Pd(II) starting material, these reactions afford either monomeric Pd(IV) complexes such as **19** (ref. 68), **20** (ref. 69) and **21** (ref. 70) or dimeric Pd(III) species such as **22** (ref. 71) (Fig. 4). Many of these high-valent palladium compounds are isolatable at room temperature (25 °C). However, on being heated, they all undergo kinetically fast and highly thermodynamically favourable carbon-heteroatom bond-forming reductive elimination to release halogenated organic products (Fig. 5a).

The stoichiometric studies shown in Fig. 5a have informed the development of new palladium-catalysed halogenation reactions that involve

high-valent intermediates. One particularly well-studied example involves the ligand-directed halogenation of arene and alkane carbon-hydrogen bonds^{62–64,72,73} (Fig. 5b). Electrophilic halogenating reagents (X^+) are used to promote the formation of high-valent palladium intermediates during

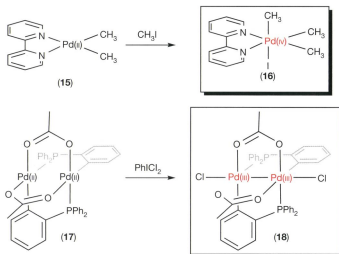


Figure 4 | Early examples of Pd(III) and Pd(IV) organometallic complexes. Ph, phenyl.

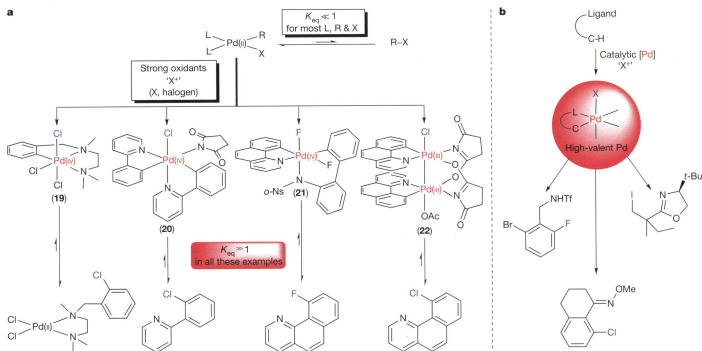


Figure 5 | High-valent palladium complexes involved in carbon-halogen bond formation. **a**, Carbon-halogen bond-forming reductive elimination is thermodynamically unfavourable for most Pd(II) species but not from high-valent palladium complexes such as **19–22**. Ac, acetyl; K_{eq} , equilibrium

constant. **b**, Select examples of palladium-catalysed carbon-hydrogen halogenation reactions. L, L-type ligand; R, aryl; X, halogen; *o*-Ns, *ortho*-nosyl; *t*-Bu, *tert*-butyl.

catalysis. Depending on the structure of the reagent, diverse carbon-halogen bonds can be formed. For example, *N*-fluoropyridinium salts generate carbon-fluorine bonds, *N*-chlorosuccinimide promotes the formation of carbon-chlorine bonds and acetyl hypiodite provides access to iodinated products.

Detailed mechanistic studies of the palladium-catalysed carbon-hydrogen chlorination of benzo[*h*]quinoline with *N*-chlorosuccinimide support the intermediacy of a high-valent palladium species⁷¹ (Fig. 6a). The resting state of the catalyst was determined to be the dinuclear succinate-bridged Pd(II) complex **23** (Fig. 6b). This compound is a kinetically competent catalyst in the presence of added acetate. Furthermore, rate studies of the **23**-catalysed carbon-hydrogen chlorination reaction show a first-order dependence on this Pd(II)–Pd(II) dimer and a first-order dependence on the oxidant. These data are consistent with rate-limiting two-electron oxidation of **23** to generate the high-valent Pd(III)–Pd(III) dimer **22** (Fig. 6b). This Pd(III)–Pd(III) intermediate could not be observed under the catalytic conditions, as to be expected when oxidation is rate limiting. However, dimer **22** could be synthesized independently at -78°C . On being warmed to 23°C , **22** underwent carbon-chlorine bond-forming reductive elimination to release chlorinated product **24** in 84% yield, further supporting its intermediacy in catalysis.

High-valent palladium catalysis has also been exploited for the halofunctionalization of alkenes^{72–74} (that is, the addition of a halogen and another functional group), as exemplified by Fig. 6c. The combination of a palladium catalyst, an alkene and an aryl–metal reagent such as Bu_3SnPh is well known to produce a Pd(II)–alkyl intermediate like **25** (Fig. 6c). However, the fate of this intermediate and the ultimate organic product of the reaction vary drastically depending on the choice of oxidant. For example, with oxidants such as dioxygen (which has low kinetic reactivity with most Pd(II) complexes), **25** undergoes β -hydride elimination to release styrene product **26** through a conventional low-valent Pd(II) or Pd(0) manifold⁷⁷ (Fig. 6c, reaction (i)). By contrast, kinetically reactive Cl^+ oxidants such as PhCl_2 can rapidly intercept **25** to generate putative high-valent palladium intermediates. These can

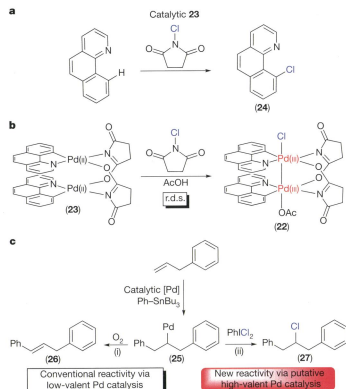


Figure 6 | Palladium-catalysed chlorination. **a**, Complex **23** is an efficient catalyst for the palladium-catalysed carbon-hydrogen chlorination of benzo[*h*]quinoline. **b**, The rate-determining step (r.d.s.) of this palladium-catalysed reaction is oxidation of **23** by *N*-chlorosuccinimide to form the Pd(III)–Pd(III) dimer **22**. **c**, High-valent palladium-catalysed 1,2-arychlorination (ii) is complementary to low-valent palladium-catalysed reactions (i) of α -alkenes.

then undergo carbon–chlorine bond-forming reductive elimination to release 1,2-arylchlorinated compound **27** (ref. 76; Fig. 6c, reaction (ii)). Although the intermediacy of Pd(III) and/or Pd(IV) in these arylchlorination reactions has not yet been definitively confirmed, the observed reactivity (favouring carbon–chlorine bond formation over β -hydride elimination) is consistent with such a mechanism. This highlights another key complementarity between catalysis by low-valent palladium (where square-planar Pd(II)-alkyl intermediates typically undergo fast decomposition through β -hydride elimination) and catalysis by high-valent palladium^{62–64,72} (where β -hydride elimination is disfavoured owing to the lack of open coordination sites at octahedral Pd(III)- and/or Pd(IV)-alkyl complexes). Notably, many related palladium-catalysed alkene difunctionalization reactions have been reported over the past six years that also probably proceed through high-valent palladium pathways^{53,64}.

Another challenging and desirable chemical target for catalysis by high-valent palladium has been the generation of carbon–CF₃ linkages. Trifluoromethyl groups appear in numerous commercial pharmaceuticals and drug candidates and can drastically enhance the metabolic stability and bioavailability of biologically active molecules⁷⁸. Despite the prevalence of these groups in medicinal chemistry, efficient approaches to introducing CF₃ into organic compounds under mild conditions are limited. Methods involving metal catalysts are particularly rare^{79,80} and would be powerful synthetic tools to complement currently available chemical processes.

Many previous efforts to develop catalytic trifluoromethylation reactions have been hampered by the kinetic inertness of most metal–CF₃ complexes to carbon–CF₃ bond formation⁷⁹. For example, carbon–CF₃ coupling at Pd(II) centres requires specialized phosphine ligands to proceed efficiently^{79,80} (Fig. 7a). By contrast, Pd(IV) complexes containing simple bidentate, nitrogen donor ligands (N–N) undergo facile carbon–CF₃ bond-forming reductive elimination. For example, the stoichiometric reaction of (N–N)Pd(II)(aryl)CF₃ complexes with *N*-fluoropyridinium oxidants affords isolatable high-valent

(N–N)Pd(IV)(aryl)CF₃ intermediates. These compounds participate in rapid carbon–CF₃ coupling at temperatures as low as 25 °C (ref. 81).

A related approach has been used to achieve catalytic ligand-directed trifluoromethylation of aromatic carbon–hydrogen bonds. In this system, electrophilic trifluoromethylating reagents (CF₃⁺) were used to promote the formation of high-valent palladium intermediates, which decompose to afford aryl-trifluoromethylated products⁸². Remarkably, these reactions proceed efficiently with simple palladium salts as catalysts, and no external ligands (other than substrate) are required. Subsequent mechanistic studies suggested that the Pd(IV) complex **28** might be a catalytic intermediate⁸³ (Fig. 7b), as it serves as a kinetically competent catalyst under the reaction conditions. This methodology represents a transformation (ligand-directed C–H $\rightarrow$ C–CF₃ conversion) that is not at present accessible using any other transition-metal catalyst, again highlighting the power of high-valent palladium to do novel chemistry.

In addition to the carbon–halogen and carbon–trifluoromethyl bond-forming reactions discussed above, high-valent palladium intermediates have also been implicated in the selective transformation of alkane and arene carbon–hydrogen bonds into carbon–oxygen, carbon–carbon, carbon–nitrogen and carbon–sulphur linkages. Detailed mechanistic investigations of catalytic carbon–hydrogen acetoxylation⁸⁴ and arylation⁸⁵ have provided evidence consistent with the formation of high-valent palladium intermediates in these reactions as well.

There has been much recent progress in high-valent palladium catalysis. Over the past decade, numerous organometallic Pd(IV) and Pd(III) complexes have been synthesized by the reaction of Pd(II) starting materials with strong oxidants. A wide range of carbon–carbon and carbon–heteroatom bond-forming reductive elimination reactions can be achieved from these species, and the selectivity, reactivity and mechanisms of these transformations have been studied in detail. Furthermore, a number of these species have been shown to be kinetically competent catalysts for carbon–hydrogen bond halogenation, trifluoromethylation and other reactions. These results have firmly established the feasibility

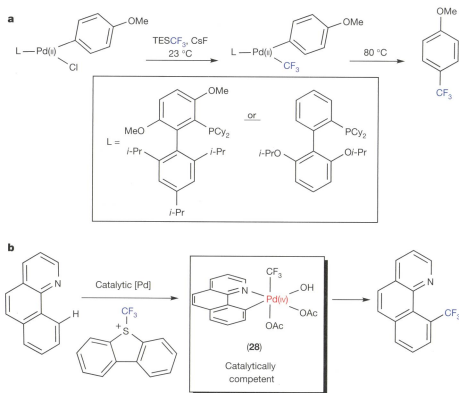


Figure 7 | Palladium-catalysed trifluoromethylation. a, Carbon–CF₃ bond formation from Pd(II) requires specialized phosphine ligands. TES, triethylsilyl; Cy, cyclohexyl; *i*-Pr = *iso*-propyl. b, Using a high-valent palladium strategy,

catalytic carbon–hydrogen trifluoromethylation has been developed through putative Pd(IV) intermediate **28**.

and synthetic utility of high-oxidation-state palladium catalysis in organic synthesis, and the field shows great potential for the future.

Comparison and contrast

As discussed here, high-valent copper chemistry has several features in common with high-valent palladium chemistry. First, although for each metal the specific transformations that have been selected for detailed mechanistic investigation vary significantly, Cu(III) and Pd(III)/Pd(IV) species have been shown to participate in closely related carbon–carbon and carbon–heteroatom bond-forming reductive elimination reactions. One particularly striking example is the accessibility of carbon–halogen bond-forming reductive elimination from Cu(III) complex **13** as well as from Pd(IV)/Pd(III) complexes **19–22**. Similar ligand environments have been shown to stabilize high-valent complexes of both metals. In particular, rigid multidentate ligands (such as the macrocycles of copper complexes **2, 10** and **13** and the cyclometalated benzo[h]quinoline of Pd(IV) species **21** and **28** and Pd(III) complex **22**) tend to slow competing reductive elimination processes. Furthermore, the presence of multiple highly electron-donating σ -aryl or σ -alkyl ligands (as in Cu(III) complexes **4–9** and Pd(IV) complexes **16** and **19–21**) facilitate the detection or isolation of high-valent species of both copper and palladium.

Recent examples of copper- or palladium-catalysed oxidation reactions reveal additional intriguing similarities. There are a multitude of catalytic carbon–carbon and carbon–heteroatom coupling reactions that share the following features: a copper or palladium catalyst, an oxidant and an organic substrate that is a precursor to a metal–carbon bond (such as an aryl halide, a carbon–hydrogen bond or a transmetalating reagent). Three examples of such transformations are shown in Fig. 8. Although detailed mechanistic analysis will be required to establish firmly the pathway for each system, it seems likely that many (if not all) of these reactions proceed through high-valent copper or palladium manifolds. In the first example, copper and palladium catalyse the same overall reaction, the ligand-directed carbon–hydrogen acetoxylation of 2-phenylpyridine^{52,65} (Fig. 8a). In the second example, the same oxidant (S-(trifluoromethyl) dibenzothioephonium) is used to effect the trifluoromethylation of two different organic substrates^{52,66} (Fig. 8b). Finally, in the third example, both metals catalyse the carbon–hydrogen arylation of indole with diaryliodonium salts^{67,68} (Fig. 8c).

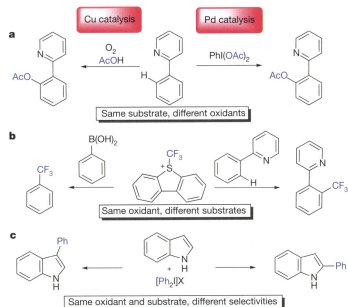


Figure 8 Oxidative bond-forming reactions catalysed by copper and palladium. These reactions exemplify similarities and differences between copper and palladium.

The reactions in Fig. 8 not only illustrate key similarities but also highlight key differences or complementarities between the oxidative chemistry of palladium and that of copper. For example, the palladium-catalysed carbon–hydrogen acetoxylation of 2-phenylpyridine (Fig. 8a) requires the use of $PhI(OAc)_2$ as the terminal oxidant⁶⁵. This reagent is quite expensive and generates an equivalent of iodobenzene waste with each catalytic turnover. By marked contrast, the copper-catalysed acetoxylation uses abundant and atom-economical dioxygen as the oxidant⁶². The ability to generate high-valent copper using dioxygen is currently a distinct advantage of high-valent copper catalysis^{40,42,53}. Although dioxygen is thermodynamically capable of oxidizing Pd(II) to Pd(IV), most organo-Pd(II) intermediates are kinetically inert to oxidation by dioxygen⁷¹. However, two recent reports have shown that aerobic, palladium-catalysed, ligand-directed carbon–hydrogen oxygenation is possible (potentially through high-valent palladium intermediates), suggesting a promising future in this area^{69,90}.

As shown in Fig. 8b, both copper and palladium catalyse carbon–CF₃ bond-forming reactions with S-(trifluoromethyl) dibenzothioephonium^{52,66}. Catalytically competent Pd(IV) intermediates have been observed and isolated in the carbon–hydrogen trifluoromethylation reaction⁶⁶ (Fig. 7b). By contrast, mechanisms involving Cu(III)(aryl)CF₃ intermediates have been proposed but remain to be confirmed experimentally for the copper-catalysed trifluoromethylation of boronic acids⁶⁶. These two examples demonstrate another key complementarity between palladium and copper catalysis. High-valent palladium catalysis has been used to transform carbon–hydrogen substrates into many different functional groups (with trifluoromethyl being just one example), and palladium-catalysed carbon–hydrogen oxidation is an extremely common, general, well-studied reaction⁷². By marked contrast, high-valent copper catalysis has predominantly focused on prefunctionalized substrates such as aryl boronic acids and aryl halides (Fig. 8b). At present, copper-catalysed carbon–hydrogen bond oxidation reactions are comparatively rare and the range of possible substrates is significantly narrower than that of analogous palladium-catalysed reactions^{70–53}. For example, whereas the copper-catalysed functionalization of unactivated alkane carbon–hydrogen bonds remains highly challenging, such transformations are increasingly common using palladium⁷³. The development of more robust and general methods for carbon–hydrogen bond oxidation through catalysis by high-valent copper is likely to be a major thrust of research in this field.

Finally, both copper and palladium catalyse the arylation of indole with diaryliodonium salts; however, the site selectivities of these two reactions are orthogonal (Fig. 8c). Whereas the palladium-catalysed reaction results in selective arylation at the 2-position⁶⁷, the copper-catalysed methods can be tuned to give exclusive arylation at the 3-position⁶⁸. Site selectivity is one of the most difficult challenges in the field of carbon–hydrogen functionalization. As such, the ability to tune selectivity as a function of the metal is of great potential synthetic utility. Thus far, neither of these transformations has been the subject of detailed mechanistic analysis, but the generation of Cu(III) and Pd(IV) intermediates has been suggested in both cases. In combination, the examples in Fig. 8 demonstrate the tremendous opportunities available in the concurrent development of high-valent copper and palladium catalysis.

Looking forward

The fields of high-valent palladium and copper chemistry are sure to have a bright and rapidly expanding future. It will be critical to increase our understanding of, and enhance the chemo-, regio- and stereoselectivity of, catalytic processes involving high-valent copper and palladium intermediates. In many cases, the coordination sphere of these high-valent metal centres contains multiple possible partners for reductive bond-forming reactions. The ability to control the chemoselectivity of the bond-forming event is of central importance in achieving efficient, high-yield catalytic transformations. In addition, the identification of chiral ligands that are compatible with catalysis by high-valent copper

and/or palladium could potentially allow novel asymmetric conjugate addition, aryl-aryl coupling, carbon-hydrogen oxidation and/or alkene difunctionalization reactions, which would all be of great value for organic synthesis.

Additional future work in this field will focus on expanding the scope of the fundamental organometallic reactions that are possible at high-valent copper and palladium centres. Despite the impressive progress described above, the synthetic power of high-valent organometallic intermediates has thus far been explored quite narrowly, with an almost exclusive focus on reductive bond-forming reactions. We anticipate that the design of new ancillary ligands that even better stabilize high-valent palladium and copper will facilitate the study and application of carbon-hydrogen activation, σ -bond metathesis, migratory insertion and nucleopalladation reactions at these metal centres. Such reactions could potentially proceed with novel patterns of reactivity and selectivity relative to analogous transformations of low-valent analogues. For example, several preliminary reports have suggested that carbon-hydrogen activation occurs with completely different site selectivities at Pd(IV) centres than at Pd(II) centres^{91–94}.

Finally, a number of recent reports suggest that high-valent organometallic complexes of other late transition metals can catalyse reactions similar to those discussed for Cu(III) and Pd(III)/Pd(IV) above. For example, complexes of nickel(III), nickel(IV), silver(II) and silver(III) have been observed and/or implicated in carbon-halogen and carbon-nitrogen bond-forming processes^{95–100}. Further exploration of these is likely to uncover many additional applications for high-valent late transition metals in catalysis.

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Emerging fungal threats to animal, plant and ecosystem health

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The past two decades have seen an increasing number of virulent infectious diseases in natural populations and managed landscapes. In both animals and plants, an unprecedented number of fungal and fungal-like diseases have recently caused some of the most severe die-offs and extinctions ever witnessed in wild species, and are jeopardizing food security. Human activity is intensifying fungal disease dispersal by modifying natural environments and thus creating new opportunities for evolution. We argue that nascent fungal infections will cause increasing attrition of biodiversity, with wider implications for human and ecosystem health, unless steps are taken to tighten biosecurity worldwide.

Emerging infectious diseases (EIDs) caused by fungi are increasingly recognized as presenting a worldwide threat to food security^{1,2} (Table 1 and Supplementary Table 1). This is not a new problem and fungi have long been known to constitute a widespread threat to plant species. Plant disease epidemics caused by fungi and the fungal-like oomycetes have altered the course of human history. In the nineteenth century, late blight led to starvation, economic ruin and the downfall of the English government during the Irish potato famine and, in the twentieth century, Dutch elm blight and chestnut blight laid bare urban and forest landscapes. The threat of plant disease has not abated, in fact it is heightened by resource-rich farming practices and exaggerated in the landscape by microbial adaptation to new ecosystems, brought about by trade and transportation³, and by climate fluctuations^{4,5}.

However, pathogenic fungi (also known as mycoses) have not been widely recognized as posing major threats to animal health. This perception is changing rapidly owing to the recent occurrence of several high-profile declines in wildlife caused by the emergence of previously unknown fungi^{6,7}. For example, during March 2007, a routine census of bats hibernating in New York State revealed mass mortalities⁸. Within a group of closely clustered caves, four species of bats were marked by a striking fungus growing on their muzzles and wing membranes, and the name 'white nose syndrome' (WNS) was coined. After the initial outbreak, the ascomycete fungus *Geomyces destructans* was shown to fulfil Koch's postulates and was described as the cause of WNS in American bat species^{9,10}. Mortalities exhibiting WNS have subsequently been found in an increasing number of bat overwintering sites and, by 2010, the infection was confirmed to have emerged in at least 115 roosts across the United States and Canada, spanning over 1,200 km (ref. 11). Bat numbers across affected sites have declined by over 70% and analyses have shown that at least one affected species, the little brown bat *Myotis lucifugus*, has a greater than 99% chance of becoming locally extinct within the next 16 years (ref. 11). Other species of bats across this region are declining as a consequence of this infection, and the prognosis for their survival and their role in supporting healthy ecosystems, is poor¹².

Cases of this sort are no longer perceived to be atypical. The probability of extinction is increasing for some species of North American bats, but another fungal infection has caused the greatest disease-driven loss of biodiversity ever documented. The skin-infecting amphibian fungus

Batrachochytrium dendrobatidis was discovered in 1997 (ref. 13) and named in 1999 (ref. 14). *B. dendrobatidis* has been shown to infect over 500 species of amphibians in 54 countries, on all continents where amphibians are found^{15,16}, and is highly pathogenic across a wide diversity of species. Studies using preserved amphibian specimens showed that the first appearance of *B. dendrobatidis* in the Americas coincided with a wave of population declines that began in southern Mexico in the 1970s and proceeded through Central America to reach the Panamanian isthmus in 2007 (ref. 17). As a consequence of the infection, some areas of central America have lost over 40% of their amphibian species¹⁸, a loss that has resulted in measurable ecosystem-level changes¹⁹. This spatiotemporal pattern has been broadly mirrored in other continents¹⁵, and ongoing reductions in amphibian diversity owing to chytridiomycosis have contributed to nearly half of all amphibian species being in decline worldwide²⁰.

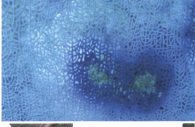
Fungal infections causing widespread population declines are not limited to crops, bats and frogs; studies show that they are emerging as pathogens across diverse taxa (Table 1), including soft corals (for example, sea-fan aspergillosis caused by *Aspergillus sydowii*)²¹, bees (the microsporidian fungus *Nosema* sp. associated with colony collapse disorder)²², and as human and wildlife pathogens in previously non-endemic regions (for example, the emergent virulent VGLI lineage of *Cryptococcus gattii* in the northwest America²³ and *Cryptococcus neoformans* across southeast Asia²⁴). The oomycetes have life histories similar to those of fungi and are also emerging as aggressive pathogens of animals, causing declines in freshwater brown crayfish (for example, the crayfish plague caused by *Aphanomyces astaci*)²⁵, Tilapia fish (for example, epizootic ulcerative syndrome caused by *A. invadans*)²⁶ and many species of plants^{27,28}. Although the direct causal relationship is uncertain in some of these diverse host–pathogen relationships, it seems that pathogenic fungi are having a pronounced effect on the global biota¹.

Increasing risk of biodiversity loss by Fungi

For infectious disease systems, theory predicts that pathogens will co-evolve with, rather than extirpate, their hosts^{29,30}. Such evolutionary dynamics mirror population-level processes in which density dependence leads to the loss of pathogens before their hosts are driven extinct³¹. For these reasons, infection has not been widely acknowledged as an

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Table 1 | Major fungal organisms posing threats to animal and plant species.

Host	Pathogen (Phylum)	Disease dynamics leading to mass mortality in animal and plant hosts
 Amphibian species (for example, the common midwife toad, <i>Alytes obstetricans</i>)	<i>Batrachochytrium dendrobatidis</i> (Chytridiomycota)	Worldwide dispersal of a hypervirulent lineage by trade ⁶⁴ . Ultra-generalist pathogen manifesting spillover between tolerant/susceptible species. Extent of chytridiomycosis is dependent on biotic and abiotic context ^{13,62} .
 Rice (<i>Oryza sativa</i>); <i>Magnaporthe oryzae</i> species complex on 50 grass and sedge species, including wheat and barley	<i>Magnaporthe oryzae</i> (Ascomycota)	Rice blast disease in 85 countries, causing 10–35% loss of harvest. Global blast population structure determined by deployment of seeds with inbred race-specific disease resistance (RSR). Invasions occur by 'host hops' and altered pathogen demographics.
 Bat spp. (little brown bats, <i>Myotis lucifugus</i>)	<i>Geomyces destructans</i> (Ascomycota)	New invasion of North American bat roosts occurred in approximately 2006, and disease is spreading rapidly ⁸ . Pathogen reservoir may exist in cave soil. Disease is more aggressive compared to similar infections in European bats, possibly owing to differences in roosts and host life histories ⁶⁵ .
 Wheat (<i>Triticum aestivum</i>); 28 <i>Puccinia graminis</i> f. <i>tritici</i> species, but <i>P. graminis</i> is found on 365 cereal or grass species	<i>Puccinia graminis</i> (Basidiomycota)	Wheat stem rust is present on six continents. Population structure is determined by deployment of RSR cultivars and long-distance spread of aeciospores. Strain Ug99 poses a notable threat to resistant wheat varieties, causing up to 100% crop loss.
 Coral species (for example, the sea fan, <i>Gorgia ventalina</i>)	<i>Aspergillus sydowii</i> (Ascomycota)	Sea-fan aspergillosis caused by a common terrestrial soil fungus ^{21,66} . Epizootics are associated with warm-temperature anomalies. Coral immunosuppression is probably a factor causing decline.
 Bee species (for example, the hive of the domestic honeybee (<i>Apis mellifera</i>) suffering colony collapse disorder)	<i>Nosema</i> species (Microsporidia)	Microsporidian fungal infections are associated with colony collapse disorder and declining populations. Pathogen prevalence is probably a part of a multifactorial phenomenon that includes environmental stressors and polyparasitism ^{57,68} .
 Sea turtle species (the loggerhead turtle, <i>Caretta caretta</i>)	<i>Fusarium solani</i> (Ascomycota)	Soil-dwelling saprotroph and phytopathogenic fungus. Infection causes hatch failure in loggerhead turtle nests and suboptimal juveniles ⁴⁴ . The disease dynamics fulfil Koch's postulates. Environmental forcing is suspected but not proven.

Images in Table 1, with permission: A. obstetricans chytridiomycosis mortalities, M.C.F.; M. oryzae, N. Talbot; WNS-affected little brown bats, A. Hicks; P. graminis, R. Mago; G. ventalina infected with A. sydowii, D. Harvell; A. mellifera hive suffering from colony collapse disorder, J. Evans; sea turtle eggs infected with F. solani, J. Diéguez-Uribeondo and A. Marco.

extinction mechanism owing to such intrinsic theoretical biotic limitations³². Inspection of species conservation databases would seem to confirm this idea. The International Union for Conservation of Nature (IUCN) red list database details threats to species worldwide, and analysis of the database has shown that of the 833 recorded species extinctions, less than 4% (31 species) were ascribed to infectious disease³³. Ecological studies on host–pathogen relationships support this finding by showing that lower parasite richness occurs in threatened host species, suggesting that parasite decline and ‘fade out’ occurs when hosts become rare³⁴. Therefore, given that macroevolutionary and ecological processes should promote diversity and prevent infectious diseases from driving their host species to extinction, we posed the question of whether we are witnessing increasing disease and extinction events driven by fungi on an increasingly large scale, or, alternatively, if there is evidence that a reporting bias has skewed our opinion of the current level of threat.

EIDs are those pathogens that are increasing in their incidence, geographic or host range, and virulence^{34,35}. Current attempts to detect EID events centre on capturing changes in the patterns of disease alerts recorded by disease monitoring programmes. ProMED (the Program for Monitoring Emerging Diseases; <http://www.promedmail.org>) and HealthMap (<http://healthmap.org>) have two approaches for detecting and monitoring outbreaks worldwide in plant and animal hosts: first, by active reporting of disease outbreaks, and second, by capturing diverse online data sources. To ascertain whether there are changing patterns of fungal disease, we reviewed all disease alerts in ProMED (1994–2010) and HealthMap (2006–10) for combinations of search terms to catalogue fungal alerts. We then classified these according to their relative proportion against the total number of disease alerts, and discriminated between plant- or animal-associated fungal pathogens (Supplementary Table 2). We also searched the primary research literature for reports in which EIDs have caused host extinction events, either at the regional scale (extirpations) or globally (Supplementary Table 3). These analyses show a number of positive trends associated with infectious fungi. Overall, fungal alerts comprise 3.5% of the ~38,000 ProMED records screened. However, over the period from 1995 to 2010, the relative proportion of fungal alerts increased in the ProMED database from 1% to 7% of the database total (Fig. 1a and Supplementary Table 2). This trend is observed for both plant-infecting (0.4% to 5.4%) and animal-infecting (0.5% to 1.4%) fungi. HealthMap shows a recent (2007–11) positive trend in the proportion of records of fungi infecting animals (0.1% to 0.3%) and plants (0.1 to 0.2%), and fungal disease alerts

were shown to occur worldwide (Fig. 1b). Web of Science literature searches and compilation of previous meta-analyses of infection-related species extinction and regional extirpation events show that fungi comprise the highest threat for both animal-host (72%) and plant-host (64%) species (Fig. 1c and Supplementary Tables 3 and 4). This effect is more pronounced for animal hosts (39 animal species affected versus 4 plant species); moreover, there is a notable increase in host loss during the second half of the twentieth century, driven mainly by the emergence of *B. dendrobatidis* (Fig. 1d). This effect is moderated after correcting for mass-species loss in regions of high epizootic loss (such as the mass extirpations of amphibians in Central America). However, fungi remain the major cause (65%) of pathogen-driven host loss after this correction. Our estimates are probably conservative owing to the cryptic nature of most disease-driven species impacts. For example, the lack of disease-related IUCN red list records is due to a lack of baseline data on the incidence of pathogens in natural systems compounded by inadequate disease diagnostics, reporting protocols and a lack of centralized recording mechanisms. Hence, the true numbers of extinctions and extirpations caused by fungi and oomycetes are likely to be greater as we have not been able to categorize the probably high levels of species loss in major plant (such as the *Phytophthora* dieback in Australia caused by *Phytophthora cinnamomi*; Supplementary Table 3) or animal outbreaks (for example, the effects of *B. dendrobatidis* emergence in the American wet tropics). We cannot discount the idea that sampling bias owing to increasing awareness of pathogenic fungi as EIDs may contribute to the patterns that we document. However, because of our observation that increases in the amount of disease caused by fungi are seen across many sources of data, including disease alerts, the peer-reviewed literature and previously noted patterns in human fungal EIDs³⁵, we believe that these trends are real. Therefore, the answer to our question seems to be that the data do indeed support the idea that fungi pose a greater threat to plant and animal biodiversity relative to other taxonomic classes of pathogen and hosts, and that this threat is increasing.

Fungal-disease dynamics leading to host extinction

Here we illustrate several key biological features of fungi that contribute to the epidemiological dynamics underlying contemporary increases in disease emergence and host extinction (Box 1).

High virulence

Fungi, like some bacterial and viral infections, can be highly lethal to naive hosts with rates of mortality approaching 100% (for example,

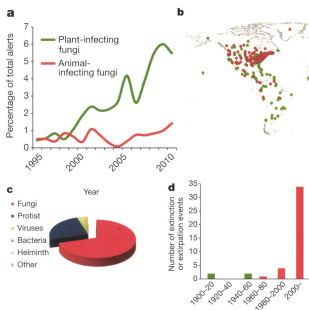


Figure 1 | Worldwide reporting trends in fungal EIDs. **a**, **b**, Disease alerts in the ProMED database for pathogenic fungi of animals and plants (**a**), and the spatial location of the associated reports (**b**). **c**, **d**, Relative proportions of species extinction and/or extirpation events for major classes of infectious disease agents (**c**) and their temporal trends for fungal pathogens (**d**). Primary data sources are given in the Supplementary Information.

BOX 1

Modelling host extinctions caused by pathogenic fungi

A simple susceptible-infected model shows that the presence of a threshold host population size for disease persistence does not prevent host extinction during a disease outbreak, especially in cases in which a lethal pathogen invades a large host population. In a large host population transmission is rapid and all hosts can become infected before the host population is suppressed below the threshold. The model follows the dynamics of susceptible (S) and infected (I) hosts during the short time duration of an epidemic (deaths that are not due to disease, and births, are ignored): $dS/dt = -\beta SI$; $dI/dt = \beta SI - \alpha I$, where β is the pathogen transmission rate, and α is the disease-induced death rate. For the parameters shown in Fig. 2a, the threshold population size below which the pathogen has a negative growth rate is $N_T = 20$ individuals. Figure 2a shows that large host populations are rapidly driven extinct, but only a fraction of individuals are killed in small host populations.

Pathogens with a long-lived infectious stage have an increased potential to cause host extinction. In this model, the disease is transmitted through contact between susceptible hosts and free-living infectious spores (Z), resulting in infected hosts, I : $dS/dt = -\beta SZ$; $dI/dt = \beta SZ - \alpha I$; $dZ/dt = \phi I - \mu Z - \beta NZ$, where β is the transmission rate, α is the pathogen-induced death rate and ϕ is the rate of release of spores from infected hosts. Figure 2b shows that fraction of hosts killed in a disease outbreak increases with the duration of the free-living infectious spore stage ($1/\mu$, where μ is the spore mortality rate).

Saprophytic growth by a pathogen can lead to extinction of the host, and even allow the pathogen to persist in the absence of its host. In this model, free-living infectious spores are released from infected hosts (with rate ϕ), and can increase in abundance through saprophytic growth, with rate σ . To illustrate the effects of saprophytic pathogen growth on host and pathogen equilibria (Fig. 2c), density-independent host reproduction (with rate b), density-dependent host mortality (with rate $d_0 + d_1 N$, where $N = S + I$), and density-dependent spore mortalities (at rate $\mu_0 + \mu_1 Z$) were included: $dS/dt = bN - (d_0 + d_1 N)S - \beta SZ$; $dI/dt = \beta SZ - \alpha I - (d_0 + d_1 N)I$; $dZ/dt = \phi I + \sigma Z - (\mu_0 + \mu_1 Z)Z - \beta NZ$.

The presence of a tolerant host species can lead to the extinction of a susceptible host species. In this model, species A is the tolerant host species, which can become infected and shed infectious spores but does not die as a result of the disease, whereas the susceptible host species (species B) has a disease-induced per-capita mortality rate of α_B . Figure 2d shows that species B is driven extinct at high densities of species A. $dS_A/dt = b_A N_A - (d_{A0} + d_{A1} N_A)S_A - \beta_A S_A Z$; $dI_A/dt = \beta_A S_A Z - (d_{A0} + d_{A1} N_A)I_A$; $dS_B/dt = b_B N_B - \beta_B S_B Z$; $dI_B/dt = \beta_B S_B Z - (d_{B0} + d_{B1} N_B)I_B - \alpha_B I_B$; $dZ/dt = \phi_A I_A + \phi_B I_B - \mu Z - \beta_B N_B Z$, where all parameters are as previously defined, but with the subscripts A or B referring to host species A or B, respectively.

B. dendrobatidis in amphibians, *G. destructans* in bats and *Ophiostoma ulmi* in elm trees). Virulence is a measure of the relative capacity of a microbe to cause damage to a host⁷⁸, and high virulence is associated with rapid intra-host growth rates, ultimately leading to rapid inter-host transmission^{77,78}. Species A has a high reproductive potential and in a large host population this effect can result in all individuals becoming infected before the population is driven to the low densities at which the pathogen can no longer spread (Fig. 2a). Thus, host extirpation can occur before density dependence limits the rate of transmission, a feature that has contributed to the mass extirpations seen in frog populations across the US Sierra Nevada mountains⁷⁹. Similarly, even if the pathogen does not drive the host to complete extinction, it may severely reduce the

population size to the point at which the species is vulnerable owing to catastrophic collapses as a result of stochastic⁸² or Allee effects⁸⁰.

Long-lived environmental stages

Fungi have remarkably resilient dispersal stages (a feature that they share with some spore-forming bacteria, such as *Bacillus anthracis*). The ability to survive independently outside of their host, as a free-living saprophyte or as durable spores in the environment, is probably the most important feature in driving the emergence of pathogenic fungi, owing to an increased risk of transporting the inocula to naive hosts (Fig. 2b)⁸¹. Furthermore, pathogenic fungi with a saprophytic stage (called saprogonous; Fig. 2c) can lead to host extirpation because their growth rate is decoupled from host densities and many fungal diseases threatening natural populations are caused by opportunistic fungi with long-lived environmental stages. Many fungi in the phylum Ascomycota are common soil organisms and are tolerant of salinity with the consequence that, when they enter the marine system through freshwater drainage, they are able to infect susceptible hosts such as corals (*A. sydowii*⁸²), sea otters (*Coccidioides immitis*⁸³) and the nests of loggerhead turtles (*Fusarium solani*⁸⁴). In terrestrial environments, potentially lethal fungi are ubiquitous, such as the causative agent of aspergillosis, *Aspergillus fumigatus*, and soil surveys have shown that *Geomyces* spp. are common soil organisms. Viable *G. destructans* has been recovered from the soil of infected bat caves⁸⁵, showing that the pathogen is able to survive and persist in infected roots when the bats are absent. Likewise, long-term persistence of fungal inoculum in the agricultural landscape is achieved by quiescent survival on plant debris, such as the spores of wheat stem rust (*Puccinia graminis*), which overwinter on straw stubble before infecting a secondary host.

Generalist pathogens and opportunistic pathogens

Although many fungi demonstrate extreme host specialization, exemplified by the gene-for-gene interactions between biotrophic fungi and their plant hosts, broad host ranges twinned with high virulence can be a lethal combination. Fungi exhibit the broadest spectrum of host ranges for any group of pathogens, and *B. dendrobatidis* and the oomycete *Phytophthora ramorum* (the cause of sudden oak death and ramorum blight) are known to infect 508 (ref. 16) and 109 (ref. 3) host species, respectively. Different host species vary in their susceptibility to infection and these differences create the potential for parasite-mediated competition when the pathogens concerned are generalists⁸⁶. Host species that can tolerate high infection loads while serving as a source of infectious stages (known as pathogen spill-over) act as community 'super spreaders' by maintaining persistent infectious stages in the system (Fig. 2d). Invasive North American signal crayfish, which tolerate infection by the oomycete *A. astaci*, force the infection into more susceptible European species that then decline⁸⁷, and similarly, although *P. ramorum* is deadly to *Notholithocarpus densiflorus* (tanoak) and several *Quercus* species⁸⁷, many of its other hosts survive infection but generate inoculum themselves for new infections. Furthermore, disease-tolerant life-history stages of otherwise susceptible species can maintain high pathogen levels leading to extinction dynamics. In chytridiomycosis, the long-lived multi-year tadpole stages of amphibians such as the mountain yellow-legged frog *Rana muscosa* and the midwife toad *Alytes obstetricans* are not killed by chytrid infections, but they can build up high loads of *B. dendrobatidis* that can infect and overwhelm juvenile metamorphs of the same species, leading to rapid population loss⁸⁸. Ultimately, when host-generalist pathogens manifest long-lived environmental stages, conditions may occur that lead to long-distance dispersal and infection of naive hosts and environments⁸.

Trade and transport promotes globalization of fungi

Fungi comprise most of the viable biomass in the air, with an average human breath containing between one and ten fungal spores⁸⁹. This ability of fungi to disperse results in some species with cosmopolitan distributions^{8,49,50}. However, these species are in the minority and it is noticeable that few fungi exhibit truly globally distributions; instead they

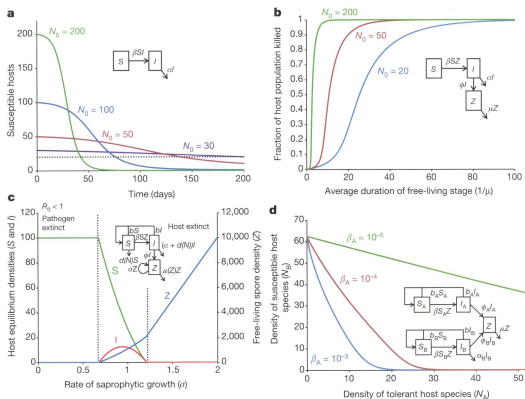


Figure 2 | Fungal disease dynamics leading to host extinction. **a**, The presence of a threshold host population size for disease persistence does not prevent host extinction during a disease outbreak, especially in cases in which a lethal pathogen invades a large host population. In a large host population transmission is rapid and all hosts can become infected before the host population is suppressed below the threshold (pathogen transmission rate, $\beta = 0.001$ per individual per day; disease-induced death rate, $\alpha = 0.02$ per day; simulations start with one infected individual and N_0 susceptible individuals). **b**, Long-lived infectious stages can increase the potential for host extinction. The fraction of hosts killed in a disease outbreak is shown as a function of the duration of the free-living infectious stage (pathogen transmission rate, $\beta = 5 \times 10^{-6}$; disease-induced death rate, $\alpha = 0.02$; rate of release of spores from infected hosts, $\phi = 10$; outbreaks initiated with one infected host in a population of N_0 susceptible individuals). **c**, Saprophytic growth: equilibrium densities of susceptible and infected hosts and free-living spores as a function of the rate of saprophytic growth, σ . With no (or low levels of) saprophytic growth, the basic reproductive rate of the pathogen (R_0) is less than 1, the pathogen cannot invade the system and the host persists at its disease-free equilibrium density. Intermediate levels of saprophytic growth allow the pathogen to invade

exhibit spatially restricted endemic ranges⁵¹. In many cases, local adaptation and host specificity are thought to underlie fungal endemicity^{51,52}. Nevertheless, when local climatic and vegetative constraints are projected globally it becomes clear that potential ranges of pathogenic fungi may be much larger than their realized range⁵³. If fungi are contained spatially by the combination of physical limits on dispersal, abiotic conditions, host distributions and genetic limits on adaptation, then how are pathogenic fungi able to overcome these barriers? Although fungi have shown the ability to undergo range expansions in response to environmental shifts⁵⁴, human-mediated intercontinental dispersal of unrecognized fungal pathogens is the major component in initiating new chains of transmission.

Pathogenic fungi have dispersed alongside early human migrations, and several thousand years ago two of these fungi, *Coccidioides immitis* and *C. neoformans* lineage VNI, seem to have invaded South America and southeast Asia, respectively, vectored by humans and their domesticated animals^{24,55}. Similar ancient patterns of human-associated disease spread are detected by studies of the genome diversity of many plant fungal pathogens⁵⁶. However, more recent increases in fungal disease are

attributable to the many-fold increase in fungal-infected trade products and food⁵⁷. The consequences of recent introductions of pathogens in association with trade are well known; examples include the Irish Famine⁴ (a consequence of *Phytophthora infestans* late blight introduction from South America), the destruction of the North American chestnuts⁵⁸ (caused by the importation of *Cryphonectria parasitica*-infected Asian chestnut trees to the east coast of the United States in the early twentieth century) and the Second World War introduction of *Heterobasidion annosum* into Italy from the USA (vectored by untreated wooden transport crates)⁵⁹. Human-mediated intercontinental trade has also been linked clearly to the spread of animal-pathogenic fungi through the transportation of infected vector species. *B. dendrobatidis* has been introduced repeatedly to naive populations worldwide as a consequence of the trade in the infected, yet disease-tolerant species such as North American bullfrogs (*Rana catesbeiana*)^{60–62} and African clawed frogs (*Xenopus laevis*)^{63,64}. Whether the emergence of bat WNS constitutes an introduction of *G. destructans* into North America from Europe or elsewhere remains to be shown. However, the widespread but apparently non-pathogenic nature of the infection in European bats tentatively

persist with the host. High levels of saprophytic growth lead to extinction of the host, and the pathogen persists in the absence of the host (host intrinsic rate of increase, $r = b - d_0 = 0.01$; density-independent host death rate, $d_0 = 1 \times 10^{-4}$; strength of density dependence in host death rate, $d_1 = 1 \times 10^{-4}$; pathogen transmission rate, $\beta = 1 \times 10^{-5}$; disease-induced death rate, $\alpha = 0.02$; rate of release of spores from infected hosts, $\phi = 10$, density-independent spore mortality rate, $\mu_0 = 1$; strength of density-dependence in spore mortality rate, $\mu_1 = 1 \times 10^{-4}$). **d**, The presence of a tolerant host species (host species A), which can become infected and shed infectious spores can lead to the extinction of a susceptible host species (host species B). Species A does not die because of the disease, but species B has a disease-induced per-capita mortality rate of α_B . Species B is driven extinct at high densities of species A. For all parameters, subscripts A or B indicate the host species. Host intrinsic rates of increase, $r_A = r_B = 0.01$; density-independent host death rates, $d_{A0} = d_{B0} = 1 \times 10^{-3}$; host birth rates, $b_A = b_B = r_A + d_{A0}$; density-independent death rate for species B, $d_{B1} = 1 \times 10^{-3}$; rate of release of spores from infected hosts, $\phi_A = \phi_B = 10$; spore mortality rate, $\mu = 1$. The density of tolerant species N_A was varied by varying d_{A1} (the strength of density-dependence in host species A), such that $N_A = r_A/d_{A1}$.

suggests that the disease may have been vectored from this region in contaminated soil⁶⁵.

Accelerated evolution of virulence in pathogenic fungi

Human activities are not only associated with the dispersal of pathogenic fungi, they also interact with key fungal characteristics, such as habitat flexibility, environmental persistence and multiple reproductive modes, to cause the emergence of disease. Importantly, many fungi are flexible in their ability to undergo genetic recombination, hybridization or horizontal gene transfer⁶⁶, causing the clonal emergence of pathogenic lineages but also allowing the formation of novel genetic diversity leading to the genesis of new pathogens^{56, 67}. Reproductive barriers in fungi are known to evolve more rapidly between sympatric lineages that are in the nascent stages of divergence than between geographically separated allopatric lineages, in a process known as reinforcement^{68, 69}. As a consequence, anthropogenic mixing of previously allopatric fungal lineages that still retain the potential for genetic exchange can drive rapid macroevolutionary change. Although many hybrids are inviable owing to genome incompatibilities, large phenotypic leaps can be achieved by the resulting 'hopeful monsters', leading to host jumps and increased virulence⁷⁰. Such mechanisms are thought to drive the formation of new pathotypes in plant pathogens⁵⁷, and oomycetes as well as fungi exhibit the genesis of new interspecific hybrids as lineages come into contact^{71, 72}. Evidence of the effect of multiple fungal co-dispersal events and recombination can also be seen in the recent *C. gatti* outbreaks in northwestern North America. In this case, strains that do not normally recombine have increased their virulence by undergoing recombination and adaptation to overcome mammalian immune responses^{23, 67}. Recent studies based on the resequencing of *B. dendrobatidis* genomes have shown that, although several lineages exist, only a single lineage (known as the *B. dendrobatidis* global panzootic lineage) has emerged in at least five continents during the twentieth century to cause epizootic amphibian declines⁶⁴. Notably, the genome of the *B. dendrobatidis* global panzootic lineage shows the hallmarks of a single hybrid origin and, when compared against other newly discovered lineages of *B. dendrobatidis*, is more pathogenic, suggesting that transmission and onward spread of the lineage has been facilitated by an increase in its virulence. Given that the rate of intra- and inter-lineage recombination among fungi will be proportional to the contact rates between previously geographically separate populations and species, these data from across plant and animal fungal pathosystems suggest that the further evolution of new races is inevitable given current rates of homogenization of previously allopatric, geographically separated, fungal lineages.

Environmental change as a driver of fungal EIDs

Climate fluctuation can be a potent cofactor in forcing changing patterns of fungal phenology⁷³ and are known to govern plant fungal EIDs. Models of climate change for the coming decades predict increases in global temperature, atmospheric CO₂, ozone and changes in humidity, rainfall and severe weather⁷⁴. For this reason, many interactions must be taken into consideration when attempting to predict the future effects of climate change on plant diseases⁷⁵. First, the physiological and spatial changes that plants may undergo in response to the various different components of climate change and the resulting effects on the pathogen⁷⁶, and second, the effects on the pathogen's physiology and dispersal external to their plant hosts⁷⁴. Frequently, however, experimental models have only taken into account one element of climate change, a common example being the free-air CO₂ enrichment (FACE) studies that model the effects of elevated atmospheric CO₂ (ref. 77). A notable result here has been rice blast severity being higher at higher CO₂ levels⁷⁸. However, although there has been a general trend for increased disease severity under simulated climate-change conditions⁷⁹, and although some species are thought to be changing their distribution in response to these changes (for example, *P. graminis*⁸⁰), other elements of climate change, such as

increased ozone, have been shown to have the opposite effect (for example, in *Puccinia recondita*⁷⁷).

Evidence for the idea that climate change has an impact on the dynamics and distribution of animal-infecting fungi is less clear-cut than that in relation to plant-infecting fungi and, although arguments have been made that warming trends may have contributed to the emergence of *B. dendrobatidis* in Central America and Europe^{81, 82}, there is active debate about these conclusions^{83, 84}. Regardless, it is clear that the disease state, chytridiomycosis, is linked to environmental factors; regional climate warming can increase the local range of the pathogen⁸ and disease risk is inversely related to rates of deforestation⁸⁵. Correlations between ecosystem change and a rise in infection by opportunistic pathogens has been proposed to account for the occurrence of coral reef declines worldwide. For example, disease caused by a variety of microbes threatens hard corals to the extent that two of the most abundant Caribbean reef-builders (staghorn and elkhorn corals) are now listed under the US Endangered Species Act. Across varied reef systems, the occurrence of warm-temperature anomalies leading to bleaching events is associated with increases in disease caused by opportunistic pathogens such as *A. sydowii*⁸⁶. In an allied colonial system, colony collapse disorder has resulted in steep declines of the European honeybee *Apis mellifera* in Europe and North America⁸⁷. These losses seem to be influenced by a mixture of aetiological agents that are fungal (for example, microsporidian (*Nosema ceranae*)), viral (for example, Kashmir bee virus and Israeli acute paralysis virus) and ectoparasitic (for example, *Varroa destructor*) in origin. So far, no single environmental cause has been identified that can account for the apparent reduction in the ability of honeybee colonies to resist these infections, and agricultural chemicals, malnutrition and modern beekeeping practices have all been suggested as potential cofactors for colony-collapse disorder⁸⁸. The increasing use of azole-based agricultural chemicals has been implicated as a factor underpinning the increase in the frequency of multiple-triazole-resistant (MTR) isolates of *A. fumigatus* infecting humans⁸⁹. The widespread agricultural use of azoles as a means of combating crop pathogens is speculated to have led to selection for MTR alleles, an idea that is supported by the recent discovery that resistance clusters onto a single lineage in Dutch populations of the fungus⁹⁰. Efforts must now be turned to integrating epidemiological studies with those on environmental change so that the many possible interactions and outcomes can be assessed, as making blanket predictions for fungal diseases is currently impossible⁹¹. The highly coordinated response to the recent outbreak of wheat stem rust (*P. graminis*, strain Ug99) is a positive step towards this goal^{77, 92}.

Fungal EIDs impact food security and ecosystem services

Impacts of fungal diseases are clearly manifested in crops and there are direct measurable economic consequences associated with die-off in forest and urban environments. Losses that are due to persistent and epidemic outbreaks of fungal and oomycete infection in rice (rice blast caused by *Magnaporthe oryzae*), wheat (rust caused by *P. graminis*), maize (smut caused by *Ustilago maydis*), potatoes (late blight caused by *P. infestans*) and soybean (rust caused by *Phakospora pachyrizii*) vary regionally but pose a current and growing threat to food security⁹³. Our estimates of loss of food are based on the 2009–10 world harvest statistics of five of our most important crops and make certain basic assumptions of caloric value and worldwide average production (Supplementary Table 1). Our calculations show that even low-level persistent disease leads to losses that, if mitigated, would be sufficient to feed 8.5% of the 7 billion humans alive in 2011. If severe epidemics in all five crops were to occur simultaneously, this would leave food sufficient for only 39% of the world's population, but the probability of such an event occurring is very low indeed.

Invasive tree diseases have caused the loss of approximately 100 million elm trees in the United Kingdom and the United States^{82, 94}, and 3.5 billion chestnut trees have succumbed to chestnut blight in the United States (Supplementary Table 5). Losses of western Canadian pine trees to the mountain pine beetle-blue-stain fungus association will result in the

release of 270 megatonnes of CO₂ over the period from 2000 to 2020, with a clearly ascribed economic cost both for the wood itself and the carbon released¹⁴. These, and other diseases such as 'sudden oak death' in California and 'foliar and twig blight' and 'dieback' on ornamental trees, woody shrubs and forestry plants in the European Union, affect ecological diversity, are costly to manage and account for huge losses of fixed CO₂. Indeed, we calculate regional losses of absorbed CO₂ to total 230–580 megatonnes for just a handful of diseases (Supplementary Table 5) with the higher figure equating to 0.069% of the global atmospheric CO₂. We have included both emerging (Jarrh dieback, sudden oak death and pine beetle–blue-stain fungus) and emergent diseases (Dutch elm blight and chestnut blight), as these represent the few examples for which informed estimates are possible. We are unable to quantify any of the many other recent emerging diseases, such as red band needle blight of pines, *Phytophthora alni* on alders or pitch pine canker on Monterey pines, owing to a lack of data and economic interest, both of which are trends that must be reversed. Assessing the economic burden of fungal mycoses in animals is a challenging task. Although the impact of fungal EIDs is manifested in domestic animal settings, particularly the amphibian trade²⁶ and in regions where virulent lineages have established²⁶, reporting mechanisms for outbreaks do not widely exist. In natural settings, valuations have recently estimated the losses to US agriculture that are the result of declines in bat populations at more than US\$3.7 billion per year (ref. 12). However, although broad ecosystem-level impacts of other fungal EIDs of wildlife are suspected, economic valuations of the ecosystem services that these species support are wholly lacking.

Mitigating fungal EIDs in animals and plants

The high socioeconomic value of crops means that detection and control of fungal diseases in agriculture far outpaces that in natural habitats. Epidemiological models have been developed to predict the risk of seasonally specific crop pathogens, allowing targeted control, and specific threats are assessed through consortia of research, governmental and global non-governmental organizations, led by the United Nations Food and Agricultural Organization (FAO), and related organizations. Scientifically led development of disease-resistant crop varieties has been mainly successful, although monocultures have in some instances vastly increased the susceptibility of harvests to highly virulent pathogens, a pertinent example being *P. graminis* Ug99. Conversely, although there have been some attempts to mitigate the fungal disease burden in wildlife *in situ*—most notably efforts to eliminate *B. dendrobatidis* in infected populations with the antifungal itraconazole²⁷ and the use of probiotic bacteria²⁸—communicable wildlife EIDs are essentially unstoppable once they have emerged. International biosecurity against the spread of plant fungal pathogens, although not perfect, is more advanced than protocols to protect against the introduction of animal-associated fungi. Fundamentally, this is the result of a financial dynamic: wildlife is not correctly valued economically, whereas crops are.

The World Organisation for Animal Health (also known as the OIE) and the FAO may be the best-placed authorities to coordinate tighter biosecurity controls for trade-associated fungal pathogens of animals. The OIE has listed *B. dendrobatidis* and the crayfish pathogen *A. astaci* in the Aquatic Animal Health Code as internationally notifiable infections, and the FAO compiles outbreak data on transboundary animal diseases using the emergency prevention information system (EMPRES-i). Similarly, the IUCN Wildlife Health Specialist Group determines policy that is specific to combating emerging wildlife disease internationally. On national scales there are a number of initiatives being deployed and in the United States the National Wildlife Health Centre has developed the national federal plan²⁹ to mitigate WNS in bats. Intensive monitoring and surveillance will be increasingly important in the coming years because predictive modelling and small-scale experiments can never fully predict future disease spread and severity. An increased political and public profile for the effects of fungal diseases in natural habitats is needed to highlight the importance of fungal disease control outside of the managed agricultural environment to

policy makers. If this occurs, then there will be more sympathy for attempts to improve the regulatory frameworks that are associated with biosecurity in international trade, as this is the most important tool to tackle both plant and animal fungal EIDs now and in the future. The monitoring of fungal inocula in wild populations should be the utmost priority and tighter control of international trade in biological material must be imposed, and with considerable haste. Inadequate biosecurity will mean that new fungal EIDs and virulent races will emerge at an increasingly destructive rate. In addition to better global monitoring and control, attention must also be turned to increasing our understanding of the interactions between hosts, pathogens and the environment, across regional and global scales. Integrated approaches encompassing theoretical and practical epidemiology, climate forecasting, genomic surveillance and monitoring molecular evolution are needed. These should be facilitated by scientists from currently disparate research fields entering into regular global discussions to develop clear and urgent strategies for working towards the elusive magic bullet for emerging fungal diseases: effective prevention and timely control.

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An elementary quantum network of single atoms in optical cavities

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Quantum networks are distributed quantum many-body systems with tailored topology and controlled information exchange. They are the backbone of distributed quantum computing architectures and quantum communication. Here we present a prototype of such a quantum network based on single atoms embedded in optical cavities. We show that atom-cavity systems form universal nodes capable of sending, receiving, storing and releasing photonic quantum information. Quantum connectivity between nodes is achieved in the conceptually most fundamental way—by the coherent exchange of a single photon. We demonstrate the faithful transfer of an atomic quantum state and the creation of entanglement between two identical nodes in separate laboratories. The non-local state that is created is manipulated by local quantum bit (qubit) rotation. This efficient cavity-based approach to quantum networking is particularly promising because it offers a clear perspective for scalability, thus paving the way towards large-scale quantum networks and their applications.

Connecting individual quantum systems via quantum channels creates a quantum network with properties profoundly different from any classical network. First, the accessible state space increases exponentially with the number of constituents. Second, the distribution of quantum states across the whole network leads to non-local correlations. Further, the quantum channels mediate long-range or even infinite-range interactions, which can be switched on and off at will. This makes quantum networks tailor-made quantum many-body systems with adjustable degrees of connectivity and arbitrary topologies, and thus powerful quantum simulators. Open questions like the scaling behaviour, percolation of entanglement¹, multi-partite entanglement^{2,3} and quantum phase transitions^{4–6} make quantum networks a prime theme of current theoretical and experimental research. Similarly, quantum networks form the basis of quantum communication and distributed quantum information processing architectures, with interactions taking the form of quantum logic gates^{7–10}.

The physical implementation of quantum networks requires suitable channels and nodes. Photonic channels are well-advanced transmitters of quantum information. Optical photons can carry quantum information over long distances with almost negligible decoherence and are compatible with existing telecommunication fibre technology. The versatility of quantum networks, however, is largely defined by the capability of the network nodes. Dedicated tasks like quantum key distribution can already be achieved using send-only emitter nodes and receive-only detector nodes¹¹. However, in order to fully exploit the capabilities of quantum networks, functional network nodes are required which are able to send, receive and store quantum information reversibly and efficiently.

The implementation and connection of quantum nodes is a major challenge, and different approaches are currently being pursued. An intensely studied example is an ensemble of gas-phase atoms^{12–14}, but the protocols for the generation of single excitations are inherently probabilistic⁹. Another strong contender is a single particle¹⁵, which allows for single-photon emission¹⁶, quantum gate operations^{7–19} and scalability²⁰. But single emitters generally exhibit weak light-matter

interaction resulting, again, in inherently probabilistic information exchange and very low success rates. In particular, the reversible quantum state mapping between a photonic channel and a single emitter in free space is highly inefficient. In their seminal work³, Cirac and co-workers therefore proposed to overcome these problems by network nodes based on single emitters embedded in optical cavities.

Here we present the experimental realization of this prototype of a quantum network. The nodes are formed by single atoms quasi-permanently trapped in optical cavities. The cavity-enhanced light-matter interaction opens up a deterministic path for interconversion of photonic and atomic quantum states. By dynamic control of coherent dark states^{8,21–24}, single photons are reversibly exchanged between distant network nodes. We demonstrate faithful quantum state transfer across a network channel and create entanglement between distant nodes. High fidelities and long coherence times are achieved by encoding the quantum information in the polarization of the photon and the atomic ground-state spin. Our results present a direct photonic link between two distant single emitters and pave the way for the realization of large-scale quantum networks.

In the following, we describe experiments in which we characterize a single network node and the connection of two nodes forming an elementary network. The two nodes are operated in independent laboratories at a distance of 21 m and are connected by an optical fibre link of 60 m length. In a first experiment, we demonstrate that single photons can be stored in and retrieved from a single-atom node preserving the photonic polarization state. Second, we show the faithful transfer of arbitrary atomic quantum states from one node to the other. Third, a maximally entangled state of the distant atoms is created with a fidelity of up to 98% and is maintained for at least 100 μ s. This coherence time exceeds the entanglement distribution time across the network link by two orders of magnitude, and translates into a maximum possible entangled node distance of a 20 km optical fibre path. Last, local unitary operations are performed on one of the nodes, resulting in rotations of the non-local bipartite state whereby different maximally entangled states are created.

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A universal quantum network node

Each node in our quantum network consists of a single neutral rubidium atom that is quasi-permanently trapped in a high-finesse optical resonator^{24,25} (see Methods). The physical parameters put our atom-cavity systems in the intermediate-coupling regime of cavity QED, with the resonators optimized for highly directional optical output into a single mode ($\geq 90\%$) that is efficiently coupled (up to 90%) to the fibre link (Fig. 1). The reversible conversion between quantum states of light and matter is enabled by the dynamic control of coherent dark states. The $5^2S_{1/2}$ hyperfine ground states $F = 1$ and $F = 2$ of the single ^{87}Rb atom are coupled in a Raman configuration formed by the vacuum mode of the cavity (vacuum Rabi frequency $2g$) and an external control laser field (Rabi frequency Ω_C) (Fig. 1). Control laser and cavity are both blue-detuned by several tens of megahertz from the transition to the excited $5^2P_{3/2}$, $F' = 1$ state which—for an ideal coherent dark state—is never populated. The atom is driven coherently between the two ground states by controlling $\Omega_C(t)$, where t is time. With the atom initially prepared in $|F = 2, m_F = 0\rangle$ and $\Omega_C(0) = 0$, a single photon is generated in the cavity mode by increasing the control laser power until $\Omega_C > 2g$. Owing to the finite resonator decay time, the single photon is immediately emitted into the output mode with its temporal wave-packet shape determined by $\Omega_C(t)$ (Fig. 2)²⁶. The single-photon character is confirmed by a measurement of the second-order correlation function $g^{(2)}(\tau)$ (Fig. 2e). The efficiency of the emission process is up to 60%. The inverse process of coherent absorption of a single photon is performed as a time-reversal of the emission process, and requires a decrease in $\Omega_C(t)$ timed to occur when a single photon arrives.

We produce single photons with a nearly time-symmetric envelope in one atom-cavity system (Fig. 2a) and coherently absorb them in the second system²⁷. After a selectable storage time, the photon is re-emitted (Fig. 2b). The overall write-read success rate is typically $(10 \pm 1)\%$ (all quoted errors are statistical). With a photon production efficiency of 60%, the storage efficiency is calculated to be 17%. The maximum efficiencies for photon absorption and emission are set by the atom-cavity coupling strength g and can approach unity for smaller cavity mode volumes and vanishing scattering losses at the cavity mirrors.

For encoding one qubit, we utilize the photonic polarization degree of freedom and the atomic ground-state spin, that is, the Zeeman state manifold in each hyperfine state (Fig. 2c and d). When applying a

π -polarized control laser field, the selection rules for electromagnetic dipole transitions ensure the faithful mapping of the polarization state of an incoming photon onto a well-defined superposition of atomic Zeeman states and vice versa for the re-emitted photon. To characterize the conversion process, we set the polarization of the incoming photon and compare it to that of the retrieved photon after storage in the atom²⁴. The reconstructed Poincaré sphere after a storage time of $2.5\ \mu\text{s}$ is shown in Fig. 2f. The average fidelity of the quantum memory, defined as the average overlap with the input state, is $F_{\text{qm}} = (92.2 \pm 0.4)\%$ for photons arriving in a $1\ \mu\text{s}$ time interval (starting at $t = 0.2\ \mu\text{s}$ in the graph in Fig. 2b, see Discussion section below). The measured fidelity is far above the classical limit²⁷ of $2/3$. This experiment is the first to prove coherent transfer of a qubit encoded in a single photon onto a single atom. The coherence time of our memory has been characterized earlier and exceeds $100\ \mu\text{s}$ (ref. 24).

Quantum state transfer between single atoms

In the next experiment, we transfer quantum information from node A to the distant node B by sending a single photon across the fibre link. The qubit to be transferred is the state of the atom in node A represented by

$$|\psi_A\rangle = \alpha|F = 1, m_F = -1\rangle + \beta|F = 1, m_F = +1\rangle$$

where α and β are normalized complex-valued amplitudes. We apply a π -polarized control laser pulse to the atom at node A, thereby generating a photon in the polarization state:

$$|\psi_{\text{photon}}\rangle = \alpha|L\rangle + \beta|R\rangle$$

Here $|L\rangle$ and $|R\rangle$ refer to the left and right circular polarization components of the photon. After emission of the photon, the atom at node A is left in the state $|F = 1, m_F = 0\rangle$ (Fig. 3a, left diagram and ref. 21). The photon is transmitted through the optical fibre to node B. The atom at node B is initially prepared in the state $|F = 1, m_F = 0\rangle$. Using the Raman scheme (Fig. 3b, right diagram) the incoming photon is absorbed and its polarization state is mapped onto the atomic state of node B, which becomes:

$$|\psi_B\rangle = \alpha|F = 2, m_F = -1\rangle + \beta|F = 2, m_F = +1\rangle$$

After absorption, an arbitrary quantum state has been successfully communicated from node A to node B. The qubit encoded in atom B

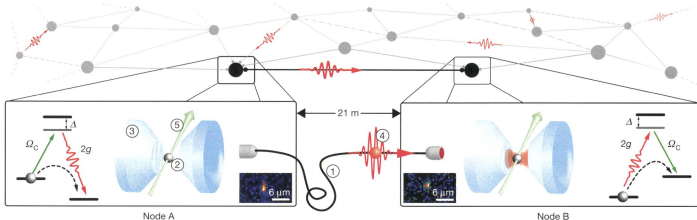


Figure 1 | A cavity-based quantum network. In the envisaged architecture (top), many single-atom nodes are connected by single-photon links. Here we explore the universal properties of the system produced by connecting two nodes (middle; A and B) within this configuration. Details of the nodes and connections are shown in the lower part of the figure. In our experiment, these two identical nodes are located in independent laboratories connected by a 60-m optical fibre (1). Each node consists of a single rubidium atom (2) trapped in an optical dipole trap at the centre of a high-finesse optical cavity (3). Quantum state transfer between the atoms and remote entanglement can be achieved via

exchange of a single photon (4), with the quantum information encoded in the internal state of the atom and the polarization of the photon. Both the production of a photon (node A) and its storage (node B) are achieved via a coherent and reversible stimulated Raman adiabatic passage (see main text for details; (5), control laser). Also shown for each node is the atomic level scheme, with the green and red arrows indicating the control laser and the exchanged single photon, respectively. Δ is the one-photon detuning. Insets, fluorescence images of the two single atoms.

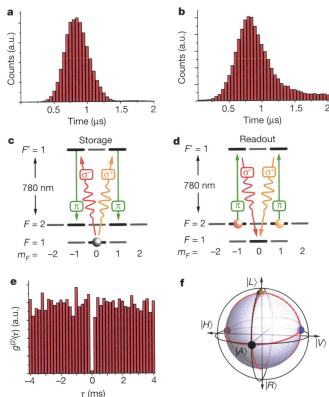


Figure 2 | Universal quantum network node. **a**, Single photons with the indicated temporal envelope (full-width at half-maximum 0.5 μ s) are produced from node A and subsequently stored in node B. **b**, Photons are retrieved after a storage time of 2.5 μ s. **c**, **d**, Atomic level schemes and optical transitions of ^{87}Rb used in the protocol. **c**, The atom at node A is initially prepared in the state $|F=1, m_F=0\rangle$ (grey sphere). The polarization of the incoming photon is a superposition of σ^+ and σ^- components (yellow and red arrows) and is converted into a superposition of the $|F=2, m_F=\pm 1\rangle$ states via a stimulated Raman adiabatic passage using a π -polarized control laser (green arrows). In the process, the phase relation between the $\sigma^\pm$ polarization components is mapped to a relative phase of the atomic Zeeman states. **d**, The photon is recreated by reversing the storage process, thereby mapping the atomic superposition state (red and yellow sphere) onto the photon's polarization. **e**, Incident photon correlations. The measured second-order correlation function $g^{(2)}(\tau)$ confirms that single photons are produced by node A. **f**, Quantum tomography of the storage process. The minimal deformation of the unit Poincaré sphere proves that every initial photonic quantum state is well preserved during storage and retrieval. The fidelity averaged over all input states is $F_{\text{QTM}} = (92.2 \pm 0.4)\%$. $|H\rangle$, $|V\rangle$, $|L\rangle$, $|R\rangle$, $|D\rangle$ and $|A\rangle$ denote horizontal, vertical, left-circular, right-circular, diagonal and anti-diagonal polarization, respectively. All quoted errors are statistical.

is identical to the original one in node A, albeit encoded in the $F=2$ hyperfine manifold. After this quantum state transfer, node A is now ready to receive another photonic qubit, whereas node B is capable of resending the stored qubit at any time. It is this symmetric and reversible feature that makes this scheme scalable to arbitrary network configurations of multiple atom-cavity systems.

We analyse the quantum state transfer using quantum process tomography²⁸. For this purpose, we prepare the atom at node A in a state of the form $|\psi_A\rangle$ by a projective measurement (see Methods). After quantum state transfer from node A to node B as described above, we read out the state of the atom in node B by mapping it onto the polarization of a second single photon which is then detected. By comparing a sufficient set of initial quantum states in node A with the obtained states in node B, we can infer the outcome of the protocol for any initial state of node A. The process matrix χ describes the mapping of the density matrix ρ_A of the state at node A (taken to be the ideally prepared state $|\psi_A\rangle$) onto the transferred state ρ_B at node B

through the operation $\rho_B = \sum_{m,n=0}^3 \chi_{mn} \sigma_m \rho_A \sigma_n^\dagger$. Here, σ_0 is the identity matrix and the other three σ_i are the usual Pauli matrices. For calculating χ we normalize the density matrices.

Ideally, the two density matrices are identical, which is equivalent to having $\chi_{00} = 1$ and all other elements zero. Figure 3c shows the absolute value of all elements of χ obtained from a maximum likelihood fit to the experimental data. We find $\chi_{00} = 0.76$ as the dominating matrix element, indicating a high level of control over the quantum process. The main deviation from a perfect state transfer is a slight depolarization of the quantum state, as indicated by the non-vanishing diagonal elements χ_{11} , χ_{22} and χ_{33} . The state transfer can also be characterized by a fidelity defined as the average overlap between initial and transferred state. We find $F_{\text{trans}} = (84 \pm 1.0)\%$, which proves the quantum character of the state transfer, as the highest fidelity achievable with classical information exchange between the nodes is 2/3. The overall success rate of the state transfer protocol is 0.2%, resulting from a production efficiency of the transmitter photon at node A of 3% (see Methods), propagation losses leading to a photon transmission of 34% and a storage efficiency at node B of about 20%.

Remote atom-atom entanglement

The most remarkable property of a quantum network is the existence of entangled quantum states shared among several network nodes. This is a basis for quantum logic gate operations between nodes and can lead to complex quantum many-body phenomena.

In the following, we demonstrate the creation of remote entanglement between distant single-atom nodes based on the transmission of a single photon. We first prepare the atom at node A in the state $|F=2, m_F=0\rangle$ (Fig. 4). Applying a π -polarized control laser pulse triggers the emission of a single photon and creates the maximally entangled state

$$|\psi_{A\otimes B}^-\rangle = \frac{1}{\sqrt{2}}(|1, -1\rangle \otimes |R\rangle - |1, 1\rangle \otimes |L\rangle)$$

between the spin state of the atom and the polarization of the photon²¹ that is routed to node B. There it is coherently absorbed and its polarization is mapped onto the spin state of atom B. The atom-photon entanglement is thus converted into entanglement between the two nodes, with the two atoms in the maximally entangled $|\Psi^-\rangle$ Bell state:

$$|\psi_{A\otimes B}^-\rangle = \frac{1}{\sqrt{2}}(|1, -1\rangle \otimes |2, 1\rangle - |1, 1\rangle \otimes |2, -1\rangle)$$

We verify the presence of this entangled state by mapping the atomic state at each node onto a photon and analysing the polarization correlations among the two read-out photons. The real part of the resulting density matrix, with the read-out performed 7 μ s after the creation of atom-atom entanglement, is shown in Fig. 4c. We find a fidelity of $F_{|\Psi^-\rangle} = (85 \pm 1.3)\%$ with the $|\Psi^-\rangle$ Bell state. This exceeds the classical limit of 50%, clearly proving the existence of entanglement between the two remote atoms. Fidelities as high as $(98.7 \pm 2.2)\%$ can be achieved by further post-selection of photon detection events (see Methods).

The success probability of entanglement creation is 2%. It is the product of the photon generation efficiency (40%) at node A, the probability with which the photon is delivered to node B (34%) and its storage efficiency at node B (14%). The verification process for the entanglement, consisting of the production of one photon at each of the two nodes and their subsequent detection, has an efficiency of 0.16%.

The entanglement created in this experiment exists between the spin states of two single atoms at a physical distance of 21 m. Highly non-classical correlations between the two atoms are observed for 100 μ s. The fidelity with the $|\Psi^-\rangle$ Bell state measured 100 μ s after creation of entanglement is $(56 \pm 3)\%$, still exceeding the classical threshold of 50% by two standard deviations. The decoherence of

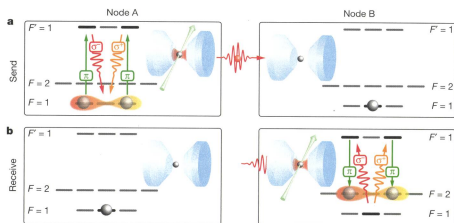


Figure 3 | Quantum state transfer between two single-atom network nodes. **a**, At node A (left), an arbitrary quantum state is encoded in the Zeeman state manifold of the single atom (see Methods). This quantum information is mapped onto the polarization of a single photon which is sent to node B (right). **b**, The photonic polarization is mapped to a superposition of atomic Zeeman

states, thereby completing the quantum state transfer from node A to node B. **c**, Absolute value of the elements of the process matrix χ for the quantum state transfer. The average fidelity between the ideal and the read-out transferred state is $(84 \pm 1)\%$, well above the classical limit of $2/3$.

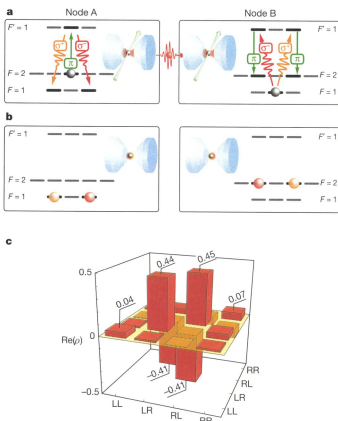


Figure 4 | Remote entanglement of two single-atom nodes. **a**, A single photon is generated at node A (left), such that the internal state of the atom and the polarization of the photon are entangled. The photon is sent to node B (right) where its polarization is mapped onto the atomic state. The grey spheres indicate the initial state of the atoms. **b**, This creates entanglement between nodes A and B that can be maintained for at least 100 μ s. The atomic levels involved in the entangled state are marked with red and yellow spheres. **c**, For analysis, both atomic states are converted into single photons. Polarization tomography on the two photons confirms the entanglement between the two nodes. We measure a fidelity of $F_{|\Psi^-\rangle} = (85 \pm 1.3)\%$ with respect to the $|\Psi^-\rangle$ Bell state. Shown is the real part of the density matrix. The magnitude of each imaginary part is ≤ 0.03 .

the atom-atom entangled state is dominated by dephasing caused by uncorrelated magnetic field fluctuations (of the order of 1 mG) at the two individual nodes and position-dependent differential a.c. Stark shifts induced by the dipole trap light fields. The dephasing due to local magnetic field fluctuations can be significantly reduced by applying small magnetic guiding fields (30 mG) along the quantization axis of each node. This has been used for the measurements with 100 μ s entanglement duration. The observed remote-entanglement lifetime exceeds the entanglement creation time (1 μ s for creation, transmission and absorption of an entangling photon) by two orders of magnitude. Entanglement lifetimes of the order of seconds can be expected when mapping the Zeeman split to magnetic-field-insensitive clock states using microwave or Raman pulses²⁹.

In the limit of unit efficiency, the entanglement scheme presented here allows for the deterministic creation of entanglement. In our experimental implementation, efficiencies are below one and we therefore detect a posteriori entanglement³⁰. The detection of entangled read-out photons indicates that atom-atom entanglement had been present. Only entanglement attempts that lead to the final detection of two read-out photons in the mapping process are considered in our data. The creation of heralded entanglement^{31–33} is possible by implementing a mechanism that signals the successful storage of a transmitted photon at node B without disturbing the stored quantum state (see Discussion below).

Local manipulation of a non-local state

Nodes A and B are in separated physical locations and thus are independently addressable for local qubit control. When two nodes are entangled, unitary operations applied locally at one of the nodes change the non-local state of both nodes while the entanglement is preserved. Thus local qubit control allows arbitrary maximally entangled two-qubit states to be created using a single initial entangled state as a resource. We demonstrate this capability by creating the $|\Psi^+\rangle$ Bell state. We start by preparing the two nodes in the $|\Psi^-\rangle$ Bell state as described above. Applying a magnetic field along the quantization axis only at node B causes a state rotation at twice the Larmor frequency. The fidelity of the created state with the $|\Psi^-\rangle$ and the $|\Psi^+\rangle$ Bell state is plotted as a function of the applied magnetic field in Fig. 5. The time between entanglement creation and read-out of the atomic state is fixed at 12.5 μ s. As can be seen from Fig. 5, the rotation of the non-local state results in a sinusoidally varying overlap with the $|\Psi^-\rangle$ Bell states. The fidelity with respect to the $|\Psi^+\rangle$ state reaches a maximum of $(81 \pm 2)\%$ at a magnetic field of $B = 30$ mG. The original $|\Psi^-\rangle$ state is recovered with a fidelity of $(76 \pm 2)\%$ after a spin

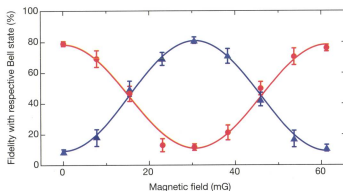


Figure 5 | Controlled rotation of the entangled state. A magnetic field, locally applied at node B, changes the non-local quantum state of the entangled nodes. The atom at node A is held at zero magnetic field. The phase evolution for a fixed hold time of 12.5 μ s is proportional to the applied magnetic field. Plotted are the fidelity with the $|\Psi^- \rangle$ and the $|\Psi^+ \rangle$ Bell state (red and blue data, respectively), showing a characteristic oscillation. The error bars indicate the statistical standard error. The solid line is a cosine fit to guide the eye. The initially prepared $|\Psi^- \rangle$ state ($F_{|\Psi^- \rangle} = (79 \pm 1.6)\%$) can be rotated into a $|\Psi^+ \rangle$ state of comparable fidelity ($F_{|\Psi^+ \rangle} = (81 \pm 2)\%$) using a magnetic field of 30 mG.

rotation of 2π . The reduced fidelity with the $|\Psi^- \rangle$ state at a field of 60 mG is a result of non-negligible Larmor precession during the entanglement creation and read-out processes.

Discussion

The body of work presented here constitutes, to our knowledge, the first direct coupling of two distant single quantum emitters by exchange of a single photon. Our results introduce universal quantum network nodes based on single emitters. Single-atom-cavity nodes excel previously investigated light-matter interfaces and incorporate their specific advantages in one platform. The use of single emitters offers a clear perspective for heralding^{34–36} and the integration of quantum gate operations both local and remote^{15,17–19,37}. In the following, we briefly discuss the potential of our specific implementation of universal quantum network nodes with respect to fidelity and efficiency of the described processes, storage time and scalability.

In all our experiments, atomic state preparation errors due to non-optimal photon pumping reduce the fidelity. Control lasers that are not perfectly π -polarized and off-resonant excitations cause deviations from the ideal transition scheme (Figs 2–4). These errors may lead to emission of photons with excitation paths different from the Raman scheme. A detailed analysis of transition strengths and effective Rabi frequencies shows that these excitation paths lead to delayed photon emission. The contribution of these photons to the measured signal can therefore be suppressed by post-selecting subsets of data based on photon arrival times (see Methods). As an example, entangled-state fidelities as high as $(98.7 \pm 2.2)\%$ are reached when considering only the first 50% of read-out photons from node A and the initial 14% of photons retrieved from node B. These results show the great potential for achieving very high fidelities if the mentioned imperfections are overcome.

The efficiency achievable with the demonstrated deterministic entanglement scheme⁹ is higher than what can be achieved with probabilistic schemes^{3,15}. Although our first implementation is not deterministic and the entanglement is not heralded, our efficiencies exceed previous demonstrations of remote entanglement by several orders of magnitude^{21–33}. The main limitation in our experiments is the moderate atom-cavity coupling strength, g . Efficiencies approaching unity may be accomplished when the cavity mode volume is decreased³⁶ and mirror scattering losses are eliminated. Ultimately, the loss of photons in the optical fibre connecting two quantum nodes will limit the overall efficiency for long distances, underlining the importance of a heralding scheme.

Heralding is crucial in networks with non-unity success probability of the quantum link, and ensures scalability in more complex and long-distance quantum networks. For architectures based on single atom-cavity nodes this is conceptually straightforward. The successful storage process is associated with a change of the atomic hyperfine ground state. The depopulation of the initial state can then be probed spectroscopically, using, for example, cavity-assisted fluorescence hyperfine state detection or cavity transmission^{34–36}. As the splitting between the two hyperfine ground states in ^{87}Rb is large, optical probing of the $F=1$ population will leave the qubit stored in $F=2$ unaltered. The main challenge is to detect $F=1$ with high fidelity before off-resonant excitation leads to decay into $F=2$, that is, with only a few scattered photons. Although the implementation of heralding is therefore challenging with our cavities, it is certainly feasible with current cavity technology³⁶.

The very weak coupling of the nuclear spin of single atoms to the environment can be exploited to boost the coherence times of our network nodes by mapping the Zeeman qubit onto magnetic-field insensitive clock states³⁹. The number of qubits per node may be increased through the use of optical lattices and single-atom registers³⁸. After the preparation of remote atom-atom entanglement using a procedure akin to that shown here, the registers in different nodes could be shifted to successively produce many sets of entangled atoms which can then be used, for example, for nested entanglement purification. This possibility, in combination with the long storage times achievable with single atoms and the potential for heralding, represents a realistic avenue towards quantum communication over arbitrary distances by means of a quantum repeater protocol¹⁰.

Current advances in photonic technologies allow reconfigurable routing between different nodes, thereby enabling various different network topologies. The controlled interaction between arbitrary nodes and the plethora of accessible topologies of cavity quantum networks are not only an important resource for quantum information processing; cavity networks also constitute a suitable paradigm for investigating emergent phenomena, such as quantum phase transitions of light⁴⁰ or percolation of entanglement⁴¹.

METHODS SUMMARY

The two independent quantum nodes are designed to operate with similar physical parameters. In each apparatus, a single ^{87}Rb atom is quasi-permanently trapped inside an optical dipole trap (potential depth $U_0/k_B = 3$ and 5 mK, where k_B is the Boltzmann constant) and held at the centre of a high-finesse optical cavity (finesse 6×10^4 , mirror distance 0.5 mm, mode waist 30 μ m). Both cavities have asymmetric mirror transmissions of $T_1 < 6$ p.p.m. and $T_2 \approx 100$ p.p.m., leading to a highly directional ($T_{\text{out}} \approx 0.9$) single output mode. Both systems produce photons on the D_2 line of ^{87}Rb at a wavelength of 780 nm. In this configuration, both atom-cavity systems operate in the intermediate-coupling regime of cavity QED (coherent atom-cavity coupling $g \approx 2\pi \times 5$ MHz, cavity field decay rate $\kappa = 2\pi \times 3$ MHz, atomic polarization decay rate $\gamma = 2\pi \times 3$ MHz). When a single atom is trapped inside each of the cavities, the experimental protocol runs at a repetition rate of 5 kHz, including optical pumping (20 μ s), photon generation (1 μ s), photon storage (1–100 μ s) and optical cooling of atomic motion (80 μ s). The necessary laser beams impinge perpendicular to the cavity axis. The presence and position of single atoms is monitored in real time by an electron multiplying CCD camera, which collects atomic fluorescence light (Fig. 1 inset). In combination with a longitudinally shiftable standing-wave dipole trap, the atoms are actively positioned at the centre of the cavity mode³⁸. Single-atom storage times are of the order of one minute.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Teneurins instruct synaptic partner matching in an olfactory map

Weizhe Hong¹, Timothy J. Mosca¹ & Liqun Luo¹

Neurons are interconnected with extraordinary precision to assemble a functional nervous system. Compared to axon guidance, far less is understood about how individual pre- and postsynaptic partners are matched. To ensure the proper relay of olfactory information in the fruitfly *Drosophila*, axons of ~50 classes of olfactory receptor neurons (ORNs) form one-to-one connections with dendrites of ~50 classes of projection neurons (PNs). Here, using genetic screens, we identified two evolutionarily conserved, epidermal growth factor (EGF)-repeat containing transmembrane Teneurin proteins, Ten-m and Ten-a, as synaptic-partner-matching molecules between PN dendrites and ORN axons. Ten-m and Ten-a are highly expressed in select PN-ORN matching pairs. Teneurin loss- and gain-of-function cause specific mismatching of select ORNs and PNs. Finally, Teneurins promote homophilic interactions *in vitro*, and Ten-m co-expression in non-partner PNs and ORNs promotes their ectopic connections *in vivo*. We propose that Teneurins instruct matching specificity between synaptic partners through homophilic attraction.

The chemoaffinity hypothesis was proposed nearly 50 years ago to explain the target specificity of regenerating optic nerves: developing neurons "must carry individual identification tags, presumably cytochemical in nature, by which they are distinguished one from another almost, in many regions, to the level of the single neuron"¹. Many molecules are now known that guide axons to their target areas^{2,3}, but few may mediate mutual selection and direct matching between individual pre- and postsynaptic partners. Here we show that the transmembrane Teneurin proteins instruct the selection of specific synaptic partners in the *Drosophila* olfactory circuit (Supplementary Fig. 1).

In *Drosophila*, individual classes of ORN axons make one-to-one connections with individual classes of second-order PN dendrites within one of ~50 discrete glomeruli in the antennal lobe. We refer to this specific one-to-one connection as PN-ORN synaptic partner matching. Olfactory circuit assembly takes place in sequential steps before sensory activity begins^{4–6}. PN dendrites first elaborate within and pattern the developing antennal lobe^{7–9}, which is followed by invasion of ORN axons^{10–14}. Importantly, re-positioning PN dendrites redirects their partner ORN axons without disrupting the connections¹⁵, suggesting that proper PN-ORN connections probably involve direct recognition and matching between partners.

Matching screens identified Ten-m and Ten-a

To identify potential PN-ORN matching molecules, we simultaneously labelled select PN dendrites and ORN axons in two colours and performed two complementary genetic screens (Fig. 1a, d). We overexpressed 410 candidate cell-surface molecules, comprising ~40% of the potential cell-recognition molecules in *Drosophila*¹⁶. In the first screen, we used *Mz19-GAL4* to label DA1, VA1d and DC3 PNs (hereafter Mz19 PNs), and *Or47b-RC2D2* to label Or47b ORNs (Fig. 1a, b). Or47b ORN axons normally project to the VA1m glomerulus and are adjacent to Mz19 PN dendrites without overlap. We overexpressed candidate cell-surface molecules only in Mz19 PNs to identify those that promoted ectopic connections between Or47b axons and Mz19 dendrites (Fig. 1a). We found that overexpression of *ten-m* (*P[G]9267*; Supplementary Fig. 2b) produced ectopic connections (Fig. 1c).

In the second screen, we labelled Mz19 PNs as above and Or88a ORNs using *Or88a-RC2D2* (Fig. 1d, e). Or88a ORN axons normally project to the VA1d glomerulus, intermingling extensively with VA1d PN dendrites (Fig. 1e). We overexpressed candidate cell-surface molecules in Mz19 PNs (Fig. 1d) as above and found that overexpression of *ten-a* (*P[G]E1914*, Supplementary Fig. 2a) partially disrupted the intermingling of Or88a axons and Mz19 dendrites (Fig. 1f).

In addition to impairing PN-ORN matching, *ten-m* and *ten-a* overexpression shifted Mz19 PN dendrite position (Fig. 1c, f). However, mismatching was not a secondary consequence of axon or dendrite mispositioning; mispositioning alone, caused by perturbation of other genes, does not alter PN-ORN matching^{9,13,15}. Furthermore, among 410 candidate molecules, only *ten-m* and *ten-a* overexpression exhibited mismatching defects, suggesting their specificity in PN-ORN matching.

Both *ten-m* and *ten-a* appear to encode type II transmembrane proteins^{17–19}. They possess highly similar domain compositions and amino acid sequences; each contains eight EGF-like and multiple YD (tyrosine-aspartate) repeats within its large carboxy-terminal extracellular domain (Fig. 1g). Ten-m and Ten-a were initially identified as tenascin-like molecules^{20,21}, but vertebrate teneurins were later identified as their true homologues based on sequence and domain similarity (Fig. 1h). Thus, we refer to Ten-m and Ten-a as *Drosophila* Teneurins. Teneurins are present in nematodes, flies and vertebrates. In human, teneurin-1 and teneurin-2 are located in chromosomal regions associated with intellectual disability¹⁷, and teneurin-4 is linked to susceptibility to bipolar disorder²².

Drosophila ten-m was originally identified as a pair-rule gene required for embryonic patterning^{21,23}, but this function was recently shown to be unrelated to *ten-m*²⁴. Teneurins were implicated in synapse development at the neuromuscular junction^{16,25} (see ref. 26), and Ten-m also regulates motor axon guidance²⁴. Neither the underlying mechanisms nor their potential roles in the central nervous system are known. Vertebrate teneurins are widely expressed in the nervous system^{18,27} and interact homophilically *in vitro*^{28,29}, suggesting their potential role as homophilic cell adhesion molecules in patterning neuronal connectivity.

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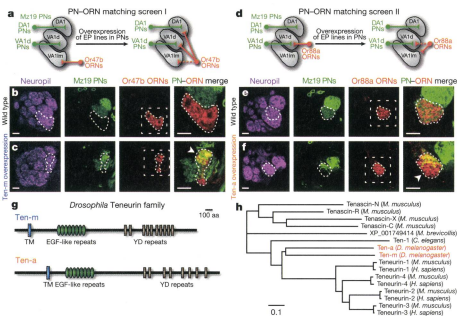


Figure 1 | PN-ORN synaptic matching screens identify two Teneurins. **a, d.** Schematics showing two PN-ORN matching screens. PN dendrites are labeled by *Mz19-GAL4* driving mCD8GFP and ORN axons by *Or47b-rCD2* (**a**) or *Or88a-rCD2* (**d**). Each EP line has a transposable element insertion that places *UAS* 5' to a gene encoding a predicted cell-surface protein, which can be overexpressed using *Mz19-GAL4*. **b, c.** *Or47b* axons and *Mz19* dendrites do not overlap in control (**b**), but form ectopic connections following *Ten-m* overexpression (**c**), as seen by axon-dendrite intermingling (arrowhead). **e, f.** *Or88a* axons and *Mz19* dendrites connect at the *VA1d* glomerulus in control (**e**), but the connection is partially lost following *Ten-a* overexpression, as some of the *Or88a* axons no longer intermingle with *Mz19* dendrites

Matching expression of *Ten-m* and *Ten-a*

Both *Drosophila* Teneurin proteins were endogenously expressed in the developing antennal lobe (Fig. 2a and Supplementary Fig. 3). At 48 h after puparium formation (APF), when individual glomeruli just become identifiable, elevated Teneurin expression was evident in select glomeruli. The subset of glomeruli expressing elevated *Ten-m* was distinct but partially overlapping with that expressing elevated *Ten-a* (Fig. 2a, e). Teneurin proteins were also detected at a low level in all glomeruli. Both basal and elevated Teneurin expressions were eliminated by pan-neuronal RNA interference (RNAi) targeting the corresponding gene (Fig. 2b, c), suggesting that Teneurin proteins are produced predominantly by neurons. In a *ten-a* null mutant we generated (Supplementary Fig. 2a), all *Ten-a* expression was eliminated, confirming antibody specificity (Fig. 2d).

The antennal lobe consists of ORN axons as well as PN and local interneuron dendrites. We used intersectional analysis to determine the cellular source for elevated Teneurin expression. For *ten-m*, we screened *GAL4* enhancer traps near the *ten-m* gene, and identified NP6588 (hereafter *ten-m-GAL4*; Supplementary Fig. 2b) that recapitulated the glomerular-specific *Ten-m* staining pattern (Supplementary Fig. 4a–c). We used a FLPout reporter *UAS>stop>mCD8GFP* to determine the intersection of *ten-m-GAL4* and an ORN-specific *ey-Flp* (Fig. 2f and Supplementary Fig. 4d–f) or a PN-specific *GH146-Flp* (Fig. 2g and Supplementary Fig. 4g–i). We found that *ten-m-GAL4* was selectively expressed in a subset of ORNs and PNs. Owing to reagent availability, we focused our analysis on five glomeruli (DA1, VA1d, VA1Im, DC3 and DA3), adjacently located on the lateral and anterior side of the antennal lobe. In these five glomeruli, *Ten-m* expression in PN and ORN classes matched: high levels in PNs corresponded to high levels in ORNs and vice versa (Fig. 2f, g).

To determine the cellular origin of elevated *Ten-a* expression, we performed tissue-specific RNAi of endogenous *Ten-a*, as no *GAL4*

(arrowhead). Target areas of *Or47b* (**b, c**) or *Or88a* (**e, f**) axons are outlined. Mismatching phenotypes are quantified in Supplementary Figs 9k and 10q. The first three columns in **b, c, e, f** show separate channels of the same section; the fourth shows higher magnification of the dashed squares (as in Figs 3, 4, 5d–g). Unless indicated, all images in this and subsequent figures are single confocal sections and all scale bars are 10 μ m. **g.** Domain composition of *Drosophila* *Ten-m* and *Ten-a*. aa, amino acids; TM, transmembrane domain. **h.** Phylogeny of the *Drosophila* Teneurins and related proteins in other species. Branch lengths represent units of substitutions per site of the sequence alignment. Teneurins are evolutionarily conserved in bilaterians and a unicellular choanoflagellate *Monosiga brevicollis*, but not in cnidarians.

enhancer trap is available near *ten-a*. To isolate *Ten-a* expression in ORNs, we drove pan-neuronal *ten-a* RNAi while specifically suppressing RNAi in ORNs using *tubP>stop>GAL80* and *ey-Flp* (Fig. 2h). To restrict *Ten-a* expression to central neurons, we expressed *ten-a* RNAi in all ORNs (Fig. 2i). We found that *Ten-a* was highly expressed in a subset of ORNs and central neurons, and also showed a matching expression in the five glomeruli we focused on (Fig. 2h, i). The glomerular-specific differential *Ten-a* expression in central neurons probably arises mainly from PNs as they target dendrites to specific glomeruli, and punctate *Ten-a* staining was observed in PN cell bodies (Supplementary Fig. 5). In summary, *Ten-m* and *Ten-a* are each highly expressed in a distinct, but partially overlapping, subset of matching ORNs and PNs (Fig. 2j).

Teneurins are required for PN-ORN matching

To examine whether Teneurins are required for proper PN-ORN matching, we performed tissue-specific RNAi (Fig. 3 and Supplementary Fig. 2c) in all neurons using *C155-GAL4*, in PNs using *GH146-GAL4*, or in ORNs using *peb-GAL4*. To label specific subsets of PN dendrites independent of *GAL4*-*UAS*, we used the Q binary expression system⁴⁰, and converted *Mz19-GAL4* to *Mz19-QF* by bacterial artificial chromosome (BAC) recombining (Supplementary Fig. 2d). We could thus perform *GAL4*-based RNAi knockdown while labelling PN dendrites and ORN axons in two colours independent of *GAL4*. We focused our analysis on *Mz19* dendrites and *Or47b* axons, which innervate neighbouring glomeruli but never intermingle in wild type (Figs 1b and 3a, b).

Pan-neuronal RNAi of both *teneurin* genes shifted *Or47b* axons to a position between two adjacent *Mz19* glomeruli, DA1 and VA1d (Fig. 3c). Moreover, *Mz19* dendrites and *Or47b* axons intermingled without a clear border (Fig. 3c, d), reflecting a PN-ORN matching defect. We confirmed this using independent RNAi lines targeting

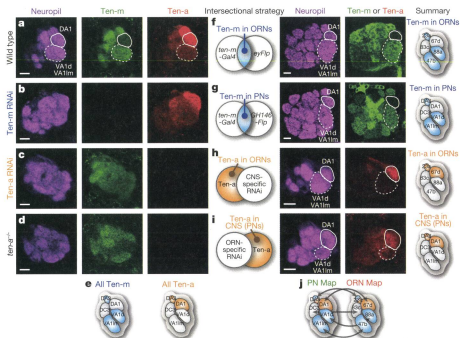


Figure 2 | Ten-m and Ten-a are differentially expressed in matching PN and ORN classes. **a**, Developing antennal lobes at 48 h APF stained by antibodies against Ten-m, Ten-a, and a neuropil marker, N-cadherin. Solid lines encircle the DA1 glomerulus (Ten-m low, Ten-a high). Dashed lines encircle the VA1d/VA1lm glomeruli (Ten-m high, Ten-a low). **b**, **c**, Ten-m and Ten-a proteins are undetectable following pan-neuronal RNAi of *ten-m* (**b**) and *ten-a* (**c**), respectively. **d**, A *ten-a* homozygous mutant eliminated the Ten-a antibody staining. **e**, Summary of elevated Ten-m and Ten-a expression in five select

glomeruli. **f**, **g**, Expression of the Flp-out GFP reporter *UAS>stop>mCD8GFP* at the intersection of *ten-m-GAL4* with ORN-specific *ey-Flp* (**f**) or with PN-specific *GHI46-Flp* (**g**) in adult. **h**, **i**, Antibody staining of Ten-a in central nervous system (CNS)-neuron-specific RNAi (**h**) or in ORN-specific RNAi (**i**) at 48 h APF. **f**–**i**, Right, individual cell-type-specific Teneurin expression patterns are schematically summarized. **j**, Combined expression patterns of Teneurin proteins in PNs (left) and ORNs (right). Blue, Ten-m high; orange, Ten-a high. Scale bars, 10 μ m.

different regions of the *ten-m* and *ten-a* transcripts (Supplementary Fig. 6). Further, knocking down *ten-m* and *ten-a* only in PNs or only in ORNs also led to Mz19–Or47b intermingling (Fig. 3e and Supplementary Fig. 7a, d), indicating that Teneurins are required in both PNs and ORNs to ensure proper matching.

Next, we examined the contribution of each Teneurin by individual RNAi knockdown in ORNs. Knocking down *ten-m* and, to a lesser extent, *ten-a*, caused mild mismatching (Fig. 3e and Supplementary Fig. 7). This was greatly enhanced by simultaneous knockdown of both *ten-m* and *ten-a* (Fig. 3e), probably because Mz19–Or47b

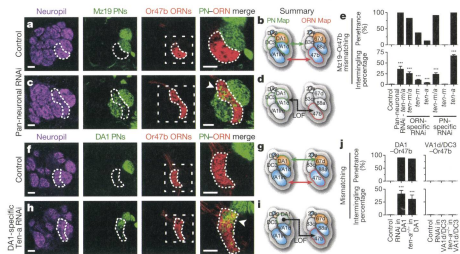


Figure 3 | Loss of Teneurins causes PN-ORN mismatching. **a**, Normally, Mz19 dendrites (green) innervate glomeruli adjacent to the VA1lm glomerulus, which is itself innervated by Or47b axons (red). The dashed line encircles Or47b axons. DC3 PNs are located posterior to DA1/VA1d PNs and Or47b ORNs, and are not visible in these sections. **c**, Mismatching phenotypes in *ten-m* and *ten-a* RNAi driven by the pan-neuronal driver *C155-GAL4*. Dashed lines encircle Or47b ORN axons, showing intermingling with Mz19 PN dendrites (arrowhead). **e**, Quantification of Mz19–Or47b mismatching phenotypes. For

all genotypes, $n \geq 15$. **f**, In control, DA1 PNs do not intermingle with Or47b ORNs. **h**, MARCM expression of *ten-a* RNAi in DA1 PNs causes dendrite intermingling with Or47b axons (arrowhead). **j**, Quantification of mismatching phenotypes. For all genotypes, $n \geq 6$. Error bars represent s.e.m. ***, $P < 0.001$ compared to control. **b**, **d**, **g**, **i**, Summary showing normal connectivity in control (**a**, **f**) and mismatching phenotypes following *teneurin* RNAi (**c**, **h**). Blue, Ten-m high; orange, Ten-a high. Green outlines, labelled PNs. Red outlines, labelled ORNs. Scale bars, 10 μ m.

mismatching requires weakening connections with their respective endogenous partners (Supplementary Fig. 7g). This synergy implies that multiple matching molecules can enhance partner matching robustness.

We also tested the functions of individual Teneurins in PNs. We found that the Mz19–Or47b mismatching was caused by PN-specific knockdown of *ten-a*, but not *ten-m* (Fig. 3e and Supplementary Fig. 7). As VA1d/DC3 and DA1 PNs arise from separate neuroblast lineages³¹, we used mosaic analysis with a repressible cell marker (MARCM) to generate neuroblast clones to label and knockdown *ten-a* in DA1 or VA1d/DC3 PNs (Fig. 3f–j; see Methods). *ten-a* knockdown only in DA1 PNs (normally Ten-a high) caused their dendrites to mismatch with Or47b axons (Fig. 3h–j). By contrast, *ten-a* knockdown in VA1d/DC3 PNs (normally Ten-a low) did not cause mismatching (Fig. 3j and Supplementary Fig. 8a, b). Similarly, MARCM loss-of-function of *ten-a* mutant in DA1 but not in VA1d/DC3 PNs resulted in mismatching with Or47b ORNs (Fig. 3j and Supplementary Fig. 8c, d). Thus, removal of *ten-a* from Ten-a-high DA1 PNs caused their dendrites to mismatch with Ten-a-low Or47b ORNs (Fig. 3i). The differential requirements of Ten-m and Ten-a in ORNs or PNs in preventing Mz19–Or47b mismatching probably reflect differential expression of Ten-m and Ten-a in the mismatching partners.

Our finding that loss of *ten-a* caused Ten-a-high PNs to mismatch with Ten-a-low ORNs (Fig. 3i, j), together with the matching expression of Teneurin proteins in PNs and ORNs, raised the possibility that Teneurins instruct scale-specific PN–ORN connections through homophilic attraction: PNs expressing high-level Ten-m or Ten-a connect to ORNs with high-level Ten-m or Ten-a, respectively.

Teneurins instruct matching specificity

This homophilic attraction hypothesis predicts that overexpression of a given Teneurin in PNs (1) should preferentially affect PNs normally

expressing low levels of that Teneurin, causing their dendrites to lose endogenous connections with their cognate ORNs, and (2) should cause these PNs to make ectopic connections with ORNs expressing high levels of that Teneurin.

To test the first prediction, we examined whether Teneurin overexpression in Mz19 PNs impaired their endogenous connections with cognate ORNs. Consistent with our prediction, Ten-m overexpression specifically disrupted the connections of DA1 PNs and Or67d ORNs, a PN–ORN pair expressing low-level Ten-m (Supplementary Fig. 9b, e). Connections of the other two pairs were unaffected (Supplementary Fig. 9a, c, d, f). Likewise, Ten-a overexpression specifically disrupted connections between VA1d PNs and Or88a ORNs, a PN–ORN pair expressing low-level Ten-a (Supplementary Fig. 9g), but not between the other two PN–ORN pairs (Supplementary Fig. 9h, i).

To test the second prediction, we examined the specificity of ectopic connections made by Mz19 PNs overexpressing Teneurins, and sampled five non-partner ORN classes that project axons to the vicinity of Mz19 dendrites (Supplementary Fig. 10). We found that Ten-m overexpression in Mz19 PNs caused their dendrites to mismatch only with Or47b ORNs (Supplementary Fig. 10f). To examine additional mismatching phenotypes that may occur within Mz19 glomeruli and to determine whether DA1 or VA1d/DC3 PNs contribute to the ectopic connections, we used MARCM to overexpress Ten-m in individual PN classes. We found that Ten-m overexpression in DA1 PNs (Ten-m low) caused their dendrites to mismatch with Or47b (Fig. 4a, b) and (to a lesser extent) Or88a ORNs (Fig. 4b, c), both endogenously expressing high-level Ten-m. By contrast, Ten-m overexpression in VA1d/DC3 PNs did not produce ectopic connections with any non-matching ORNs tested (Fig. 4d–f).

Likewise, Ten-a overexpression in Mz19 PNs caused their dendrites to mismatch only with Or23a ORNs among all non-matching ORN classes sampled outside the Mz19 region (Supplementary Fig. 10l).

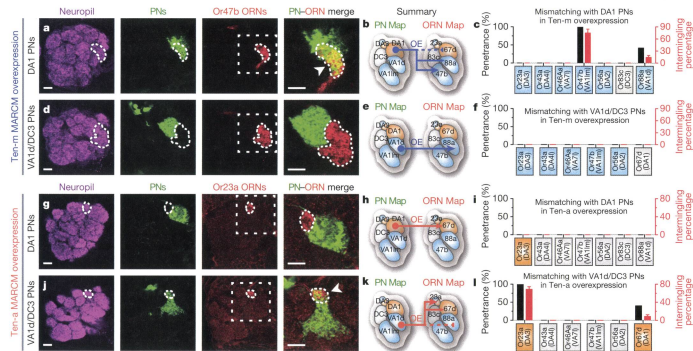


Figure 4 | Teneurin overexpression in specific PN classes causes mismatching. **a–l**, Mismatching phenotypes following Ten-m (**a–f**) or Ten-a (**g–l**) overexpression in different PN classes. Specific PN classes are labelled by MARCM with Mz19-*GAL4* and ORN axons using Or47b-*rCD2* (**a, d**) or Or23a-*mCD8GFP* (**g, j**). In control, Mz19 PNs do not intermingle with Or47b ORNs (Fig. 1b). MARCM overexpression of Ten-m in DA1 PNs (**a**, arrowhead), but not VA1d/DC3 PNs (**d**), causes dendrite mismatching with Or47b axons. MARCM overexpression of Ten-a in VA1d/DC3 PNs (**j**, arrowhead), but not in

DA1 PNs (**g**), causes their dendrites to mismatch with Or23a axons. *P[GS]9267* and *P[GE]1914* (Supplementary Fig. 2) are used to overexpress Ten-m and Ten-a, respectively. **c, f, i, k**, Quantification of mismatching phenotypes ($n = 9$ for each). Error bars represent s.e.m. See Supplementary Fig. 10 for details on some genotypes quantified here. **b, e, h, k**, Schematic summarizing the mismatching phenotypes in Fig. 4 and Supplementary Figs 9 and 10. Blue, Ten-m high; orange, Ten-a high. Scale bars, 10 μ m.

Further, MARCM overexpression of Ten-a in VA1d/DC3 PNs (Ten-a low) caused their dendrites to mismatch specifically with Or23a (Fig. 4j, k) and (to a lesser extent) Or67d ORNs (Fig. 4k, l), both endogenously expressing high-level Ten-a (Fig. 4l). By contrast, Ten-a overexpression in DA1 PNs (Ten-a high) did not produce ectopic connections with any non-matching ORNs tested (Fig. 4g–i). Thus, both Ten-m and Ten-a overexpression analyses support the homophilic attraction hypothesis.

Our data also suggest that additional molecule(s) are required to determine completely the wiring specificity of the five PN–ORN pairs examined. For example, VA1d–Or88a and VA1Im–Or47b have indistinguishable Ten-m/Ten-a expression patterns (Fig. 2j), and may require additional molecules to distinguish target choice. Indeed, Ten-a knockdown (Fig. 3h–j and Supplementary Fig. 8e, f) or Ten-m overexpression (Fig. 4b, c) caused DA1 PNs to mismatch preferentially with Or47b as opposed to Or88a axons. This suggests that the non-adjacent DA1 and VA1Im share a more similar Teneurin-independent cell-surface code than the adjacent VA1d and VA1Im. Likewise, Ten-a overexpression caused VA1d PNs to mismatch with the non-adjacent Or23a more so than the adjacent Or67d ORNs, even though both ORNs express high-level Ten-a (Fig. 4k, l). Finally, Ten-m overexpression in DC3 PNs, which express low-level Ten-m, did not change its matching specificity (Fig. 4f and Supplementary Fig. 9f), suggesting that Teneurin-independent mechanisms are involved in matching DC3 PNs and Or83c ORNs.

In summary, we showed that Teneurin overexpression in Teneurin-low PNs caused their dendrites to lose endogenous connections with Teneurin-low ORNs and mismatch with Teneurin-high ORNs (Fig. 4b, k). However, Teneurin overexpression in Teneurin-high PNs did not disrupt their proper connections (Fig. 4e, h). These data indicate that Teneurins instruct connection specificity, probably through homophilic attraction, by matching Ten-m or Ten-a levels in PN and ORN partners.

Ten-m promotes PN–ORN homophilic attractions

To test whether Teneurins interact *in vitro*, we separately transfected two populations of *Drosophila* S2 cells with Flag- and haemagglutinin (HA)-tagged Teneurins, and performed co-immunoprecipitations from lysates of these cells after mixing. We detected strong homophilic interactions between Flag- and HA-tagged Ten-m proteins and, to a lesser extent, between Flag- and HA-tagged Ten-a proteins (Fig. 5a). Ten-m and Ten-a also exhibited heterophilic interactions (Fig. 5a), which may account for their role in synapse organization²⁶.

Next, we tested whether Teneurins can homophilically promote *in vivo* trans-cellular interactions between PN dendrites and ORN axons. We simultaneously overexpressed Ten-m in Mz19 PNs using *Mz19-QF*, and Or67a and Or49a ORNs using *AM29-GAL4* (ref. 32; Fig. 5b). This enabled us to label and manipulate independently Mz19 dendrites and AM29 axons with distinct markers and transgenes. We chose *AM29-GAL4* because of its early onset of expression, whereas other class-specific ORN drivers start to express only after PN–ORN connection is established³⁴. AM29 axons do not normally connect with Mz19 dendrites (Fig. 5c, d).

Simultaneous overexpression of Ten-m in both Mz19 PNs and AM29 ORNs produced ectopic connections between them (Fig. 5c, g), suggesting that Ten-m homophilically promotes PN–ORN attraction. By contrast, Ten-m overexpression only in PNs or ORNs did not produce any ectopic connections, despite causing dendrite or axon mistargeting, respectively (Fig. 5c, e, f). These data ruled out the involvement of heterophilic partners in Ten-m-mediated attraction. Simultaneous overexpression of Ten-a in Mz19 PNs and AM29 ORNs did not produce ectopic connections (data not shown), possibly due to lower expression or weaker Ten-a homophilic interactions (Fig. 5a). Although heterophilic interactions between Ten-m and Ten-a also occur *in vitro* (Fig. 5a), heterophilic overexpression of Ten-m and

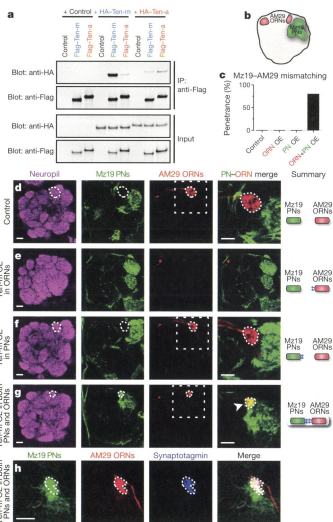


Figure 5 | Ten-m promotes homophilic interactions *in vitro* and *in vivo*.

a, Co-immunoprecipitation of Flag- and HA-tagged Teneurin proteins from separately transfected S2 cells. Co-immunoprecipitated HA-tagged Teneurin proteins are detected by anti-HA antibody and immunoprecipitated Flag-tagged Teneurin proteins by anti-Flag antibody (top two blots). The input lysates are immunoblotted for HA and Flag (bottom two blots). **b**, Schematic showing the relative positions of glomeruli targeted by Mz19 PN dendrites (green) and AM29 ORN axons (red). **c**, Quantification of mismatching between Mz19 PNs and AM29 ORNs (n = 10 for each condition). OE, overexpression. **d**, In control, Mz19 PNs and AM29 ORNs do not connect with AM29 axons. **e, f**, Overexpression of Ten-m only in AM29 ORNs (e) or Mz19 PNs (f) does not produce mismatching between them. Following Ten-m overexpression, AM29 axons mistarget posteriorly to Mz19 dendrites and are therefore not visible in e, g. Simultaneous overexpression of Ten-m in both PNs and ORNs produces ectopic Mz19–AM29 connections (arrowhead). Schematics on the right show the Mz19–AM29 connectivity in different conditions. **h**, The synaptic vesicle marker Synaptotagmin is enriched at these Mz19–AM29 ectopic connections. AM29 axons are labelled by *AM29-Gal4* with *UAS-mtd1* to visualize the entire axonal processes and *UAS-synaptotagmin-HA* to visualize synaptic vesicles in axon terminals. Mz19 dendrites are labelled by *Mz19-QF* driving *QUAS-mCD8GFP*. To overexpress Ten-m, *P[GSG]9267* and *QUAS-ten-m* (Supplementary Fig. 2) are driven by *AM29-GAL4* and *Mz19-QF*, respectively. Scale bars, 10 μ m.

Ten-a in AM29 ORNs and Mz19 PNs did not produce ectopic connections (data not shown).

Finally, we examined whether these ectopic connections lead to the formation of synaptic structures. Indeed, the ectopic connections between Mz19 dendrites and AM29 axons were enriched in synaptotagmin–HA expressed from AM29 ORNs (Fig. 5h), suggesting that these connections can aggregate synaptic vesicles and could

be functional. We propose that Teneurins promote attraction between PN-ORN synaptic partners through homophilic interactions, eventually leading to synaptic connections.

Discussion

Compared to axon guidance, relatively little is known about synaptic target selection mechanisms^{2–4}. Among the notable examples, the graded expressions of vertebrate EphA and ephrin-A instruct the topographic targeting of retinal ganglion cell axons^{43–35}. Chick DSCAM and Sidekick promote lamina-specific arborization of retinal neurons³⁶. *Drosophila* Capricious promotes target specificity of photoreceptor and motor axons^{16,37–39}. *Caenorhabditis elegans* SYG-1 and SYG-2 specify synapse location through interaction between pre-synaptic axons and intermediate guidepost cells⁴⁰. However, it is unclear whether any of these molecules mediate direct, selective interactions between individual pre- and postsynaptic partners. Indeed, in complex neural circuits, it is not clear a priori whether molecular determinants mediate such interactions. For example, the final retinotopic map is thought to result from both ephrin signalling and spontaneous activity^{41,42}. Mammalian ORN axon targeting involves extensive axon-axon interactions through activity-dependent and independent modes^{43,44}, with minimal participation of post-synaptic neurons identified thus far.

Here, we show that Teneurins instruct PN-ORN matching through homophilic attraction. Although each glomerulus contains many synapses between cognate ORNs and PNs, these synapses transmit the same information and can be considered identical with regard to specificity. Thus, Teneurins represent a strong case in determining connection specificity directly between pre- and postsynaptic neurons. We further demonstrate that molecular determinants can instruct connection specificity of a moderately complex circuit at the level of individual synapses.

Our study reveals a requirement for PN-ORN attraction in the stepwise assembly of the olfactory circuit. PN dendrites and ORN axons first independently project to appropriate regions using global cues, dendrite-dendrite and axon-axon interactions^{9,12–14}. The initial, independent targeting of PN dendrites and ORN axons is eventually coordinated in their final one-to-one matching. We identified Teneurins as the first molecules to mediate this matching process, through direct PN-ORN attraction. Our analyses have focused on a subset of PN-ORN pairs involving trichoid ORNs⁴⁵, including Or67d, Or88a and Or47b that have been implicated in pheromone sensation⁴⁶. The partially overlapping expressions of Teneurins in other PN and ORN classes (Fig. 2 and Supplementary Fig. 4) suggest a broader involvement of Teneurins. At the same time, additional cell-surface molecules are also needed to determine completely connection specificity of all 50 PN-ORN pairs.

Teneurins are present throughout Animalia (Fig. 1h). Different vertebrate teneurins are broadly expressed in distinct and partially overlapping patterns in the nervous system¹⁸. Teneurin-3 is expressed in the visual system and is required for ipsilateral retinogeniculate projections⁴⁷. Our study suggests that differential Teneurin expression may have a general role in matching pre- and postsynaptic partners. Indeed, high-level Ten-m is involved in matching select motor neurons and muscles²⁶. Furthermore, Ten-m and Ten-a also trans-synaptically mediate neuromuscular synapse organization²⁶. This suggests that the synapse partner matching function of Teneurins may have evolved from their basal role in synapse organization. Interestingly, synaptic partner matching only involves homophilic interactions (this study and ref. 26), whereas synapse organization preferentially involves heterophilic interactions²⁶. This could not be fully accounted for by the different strength of their homophilic and heterophilic interactions *in vitro* (Fig. 5a). We speculate that these dual functions of Teneurins *in vivo* may engage signalling mechanisms that further distinguish homophilic versus heterophilic interactions.

METHODS SUMMARY

Detailed methods on fly stocks, generation of the *ten-a* allele, construction of transgenic flies, clonal analysis, histology, imaging, quantification and statistical analysis, epitope-tagged constructs, and co-immunoprecipitation can be found in Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions W.H. designed and performed all experiments. T.J.M. assisted in some experiments. L.L. supervised the project. W.H. and L.L. wrote the manuscript with feedback from T.J.M.

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Structure of the mitotic checkpoint complex

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In mitosis, the spindle assembly checkpoint (SAC) ensures genome stability by delaying chromosome segregation until all sister chromatids have achieved bipolar attachment to the mitotic spindle. The SAC is imposed by the mitotic checkpoint complex (MCC), whose assembly is catalysed by unattached chromosomes and which binds and inhibits the anaphase-promoting complex/cyclosome (APC/C), the E3 ubiquitin ligase that initiates chromosome segregation. Here, using the crystal structure of *Schizosaccharomyces pombe* MCC (a complex of mitotic spindle assembly checkpoint proteins Mad2, Mad3 and APC/C co-activator protein Cdc20), we reveal the molecular basis of MCC-mediated APC/C inhibition and the regulation of MCC assembly. The MCC inhibits the APC/C by obstructing degron recognition sites on Cdc20 (the substrate recruitment subunit of the APC/C) and displacing Cdc20 to disrupt formation of a bipartite D-box receptor with the APC/C subunit Apc10. Mad2, in the closed conformation (C-Mad2), stabilizes the complex by optimally positioning the Mad3 KEN-box degron to bind Cdc20. Mad3 and p31^{comet} (also known as MAD2L1-binding protein) compete for the same C-Mad2 interface, which explains how p31^{comet} disrupts MCC assembly to antagonize the SAC. This study shows how APC/C inhibition is coupled to degron recognition by co-activators.

The fidelity of chromosome separation in mitosis is governed by an evolutionarily conserved cell-cycle checkpoint mechanism called the SAC^{1,2}. The SAC arrests the mitotically dividing cell to allow complete chromosome attachment to the bipolar mitotic spindle. The essence of the SAC is to block the onset of anaphase by inhibiting APC/C-mediated ubiquitin-dependent degradation of securin and mitotic cyclin. Components of the SAC that are responsible for detecting unattached kinetochores and for propagating signals to the APC/C have been identified^{3,4}, but the molecular basis underlying these processes is only partially understood^{1,2}. Mad2 and Mad3 (BubR1 in metazoans) mediate APC/C inhibition through their association with its co-activator subunit Cdc20 (refs 5–12). Mad2, Mad3 and Cdc20 (together with mitotic checkpoint protein Bub3) form the MCC that directly binds the APC/C to inhibit substrate recognition^{13,14}. Mad2 and Mad3 cooperate to antagonize Cdc20-dependent activation of the APC/C¹², with Mad3–Cdc20 interactions requiring the pre-assembly of a Cdc20–Mad2 complex^{12,15–18}. Thus, SAC signalling occurs through the generation of the Cdc20–Mad2 complex, a process initiated by Mad1, which is the Mad2 receptor at unattached kinetochores. Central to the association of Mad2 with Cdc20 is the inter-conversion of Mad2 between the open (O-Mad2) and closed (C-Mad2) structural states^{11,19}. These states of Mad2 differ in the topology of a carboxy-terminal β -sheet that repositions in C-Mad2 to enable binding to its protein ligands, Mad1 or Cdc20 (refs 20–22). In the template model for SAC activation, Mad1 interacts with C-Mad2, generating the C-Mad2–Mad1 complex that subsequently recruits O-Mad2 through the C-Mad2 dimerization interface. By inducing the conformational transition of O-Mad2 to C-Mad2, the Mad1-bound C-Mad2 subunit catalyses the binding of Mad2 to Cdc20 (refs 17, 23).

APC/C activity and its substrate recruitment are dependent on its co-activators (Cdc20 and Cdh1)²⁴, which recognize APC/C substrates through two destruction motifs (degrons): the D box²⁵ and the KEN (Lys–Glu–Asn) box²⁶. Mad3 contains a KEN box that is essential for MCC assembly^{15,27,28}, suggesting that Mad3 may act as a pseudosubstrate

to block substrate recognition by APC/C^{Cdc20}. However, other studies showing that the promotion of ubiquitin-mediated degradation of Cdc20 by the SAC is dependent on the KEN box of Mad3 (refs 18, 27, 29) indicate that there is a more complex mechanism controlling APC/C^{Cdc20} activity. The mechanisms underlying APC/C activation after SAC silencing are also poorly understood. In metazoans, p31^{comet} antagonizes the SAC²⁹ by functioning as a structural mimic of Mad2 that binds at the Mad2 dimerization interface to inhibit the conformational activation of O-Mad2 (ref. 31). UbcH10, assisted by p31^{comet}, catalyses Cdc20 ubiquitination, which leads to the disassembly of the MCC³².

To understand the molecular mechanisms underlying the mitotic checkpoint complex, we determined the crystal structure of the fission yeast MCC. The structure shows how Mad2 and Mad3 cooperatively inhibit Cdc20, and indicates how p31^{comet} would antagonize MCC assembly. The structure of Cdc20 in the context of the MCC offers the first opportunity to visualize degron recognition by co-activators. The interaction between Mad2 and Mad3 positions the Mad3 KEN box towards the KEN-box receptor of the Cdc20 WD40 domain. Additionally, an unexpected D-box mimic of the Mad3 C terminus reveals the D-box-binding site on Cdc20, thus demonstrating the structural basis of D-box recognition by co-activators.

Overall structure of the MCC

We generated the fission yeast MCC by co-expressing Cdc20, Mad2 and Mad3 in insect cells. The complex comprises Cdc20 with all functional domains (C box, Mad2-binding motif, WD40 domain and Ile–Arg tail (Supplementary Fig. 1)), Mad2 locked in its closed conformation that facilitates binding to Cdc20 (ref. 31), and Mad3 truncated after the tetrapeptide repeat (TPR) domain³³ and thus lacking its C-terminal KEN box. Bub3 was omitted from the complex because previous studies indicated that Bub3 was not an integral part of MCC in fission yeast²⁸ and was not required for MCC-mediated inhibition of human APC/C³⁴.

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The crystal structure of the MCC, at 2.3 Å resolution, shows that Cdc20, Mad2 and Mad3 assemble into a triangular heterotrimer (Fig. 1 and Supplementary Table 1). Mad3 coordinates the overall organization of the complex by forming numerous inter-subunit interactions with Mad2 and Cdc20, whereas Cdc20 and Mad2 interact primarily through the sequestering of the Mad2-binding motif of Cdc20 by the Mad2 'safety belt' (refs 21, 22). The core architecture of Mad3 is a contiguous TPR superhelix with three TPR motifs flanked by capping α -helices that resembles the BubR1 amino terminus³⁷. In addition, not present in the BubR1 structure, the conserved N terminus of Mad3 that incorporates the N-terminal KEN box that is essential for MCC assembly^{15,27,28} adopts a helix-loop-helix (HLH) motif (Fig. 1 and Supplementary Fig. 2). The HLH motif simultaneously binds Mad2 and Cdc20, orienting the KEN box towards its receptor on Cdc20 (Fig. 1a, b). Mad3 also contacts Mad2 and Cdc20 through its TPR domain (Fig. 1a, c).

The WD40 domain of Cdc20 conforms to a canonical seven-bladed β -propeller (Fig. 1a). The Mad2-binding motif, N-terminal to the WD40 domain, is structurally well defined, engaging the safety belt^{21,22} of Mad2 and adopting a conformation that is similar to the Mad2-binding motif of Mad1 bound to Mad2 (ref. 22) (Fig. 2a). The linker connecting the Mad2-binding motif with the N terminus of the WD40 domain is disordered, as are the two APC/C interacting motifs: a conserved Cbox that is immediately N-terminal to the Mad2-binding motif⁵, and the C-terminal Ile-Arg tail that interacts with the TPR subunit Cdc27³⁶ (Supplementary Fig. 1).

Mad2 is an α/β -HORMA-class protein²⁰ (Fig. 1a and Supplementary Fig. 3). Its C-Mad2 conformation enables interactions with the

Mad2-binding motifs of Cdc20 and Mad1 and, as revealed in the MCC crystal structure, is the only state of Mad2 that can recognize Mad3. Mad2 interacts through its α -C helix and β 8'- β 8'' hairpin with the HLH motif of Mad3 (Fig. 2b). Notably, such Mad2 interactions resemble those between C-Mad2 and p31^{comet} (ref. 31.) (Fig. 2c), and C-Mad2 in an asymmetric C-Mad2-O-Mad2 dimer²⁷ (Fig. 2d). The involvement of the β 8'- β 8'' hairpin—a region of Mad2 that undergoes substantial conformational change upon transition from the open to closed states²¹—at the Mad2-Mad3 interface indicates that Mad3 binds exclusively to C-Mad2.

Mutations of the Mad2 α -C helix disrupt C-Mad2-Mad3 interactions³⁸, consistent with our structure. To test the Mad2 interface, we replaced Met 13 with Arg (Fig. 2b). This mutation dissociated Mad3 from a Cdc20-C-Mad2 heterodimer when size-exclusion chromatography was performed (Supplementary Fig. 4). Together, these data confirm the physiological relevance of the C-Mad2-Mad3 interface and show that C-Mad2 is required to confer high-affinity binding of Mad3 to Cdc20, consistent with studies showing that Mad3 association with Cdc20 *in vivo* is synergistic with Mad2 (refs 12, 15, 16).

The Cdc20 WD40 domain is a receptor for KEN and D box

We identified two highly conserved surfaces on the Cdc20 WD40 domain, and these are responsible for APC/C degron recognition: the KEN-box²⁶ receptor, situated on the top side of the WD40 domain at the centre of the β -propeller (Fig. 3a), and the D-box²⁵ co-receptor lying in a channel between blades 1 and 7 (Fig. 4a). The KEN-box residues of Mad3 (Lys 20, Glu 21 and Asn 22) emerge from the C

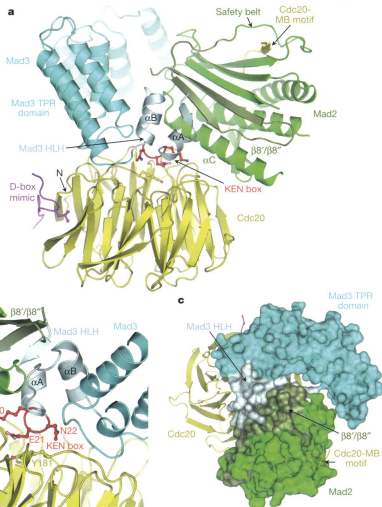


Figure 1 | Structure of *S. pombe* MCC trimer.

a, Cartoon representation of the complex of Mad2 (green), Mad3 (cyan) and Cdc20 (yellow). Mad2 is in the C-Mad2 conformation. The KEN box is shown in red, located in the HLH motif of Mad3. The D-box mimic (magenta) that is bound to Cdc20 is from the C terminus of Mad3 from a symmetry-related molecule. The Mad2 β 8'- β 8'' hairpin (dark green) that forms the Mad2-Mad3 interface, repositions on conversion from O-Mad2 to C-Mad2. The N terminus of the WD40 domain is indicated. **b**, Details of the Mad3 HLH interaction with Mad2 and Cdc20. **c**, Surface representation of the MCC. The interaction between Mad2 and Mad3 positions the Mad3 KEN box at the centre of the top side of Cdc20's WD40 domain. MB, Mad2-binding.

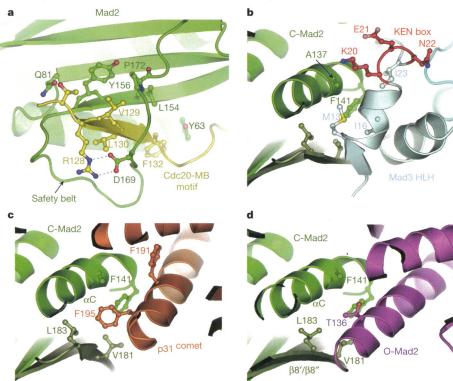


Figure 2 | Details of Cdc20–Mad2–Mad3 interactions. **a**, Mad2-binding motif of Cdc20 bound to the Mad2 safety belt. **b**, A universal Mad2 dimer interface contacts Mad3, p31^{comet} and O-Mad2. Mad2–Mad3 interactions. **c**, Mad2–p31^{comet} (ref. 31) (Protein Data Bank (PDB) accession 2QYF). **d**, C-Mad2–O-Mad2 (ref. 37) (PDB accession 2V64).

terminus of the α -A helix of the HLH as an underwound turn of α -helix (Figs 1 and 3b). Their conformation resembles a 'U', with Glu 21 at the turn dipping into a depression at the centre of the Cdc20 β -propeller toroid. Tyr 181 of Cdc20, situated on an extended loop connecting blade 1 with blade 7, acts as a platform to support the aliphatic moiety of the Glu 21 side chain. Apart from this single hydrophobic interaction, the KEN box forms entirely polar contacts involving its side-chain and main-chain groups with conserved polar and charged residues of loops on four blades of the Cdc20 β -propeller. Notably, the five residues of Cdc20 that contribute side-chain interactions to the KEN box (Asp 180, Asn 326, Thr 368, Gln 392

and Arg 438) are invariant in both Cdc20 and Cdh1, indicating a universal mode of co-activator–KEN box interaction (Supplementary Fig. 5). A proline (Pro 25) positioned three residues C-terminal to the Mad3 KEN box—well conserved in KEN-box motifs—acts to break the α -helix and orient the polypeptide chain away from the Cdc20 surface.

The Mad3 KEN box has been proposed to function as a pseudo-substrate inhibitor, blocking access of Cdc20 to KEN-box degrons in APC/C substrates¹⁵. Thus, the mode of binding of the Mad3 KEN box to Cdc20 should serve as a model for understanding how co-activators recognize APC/C substrates. Consistent with this idea, mutating the

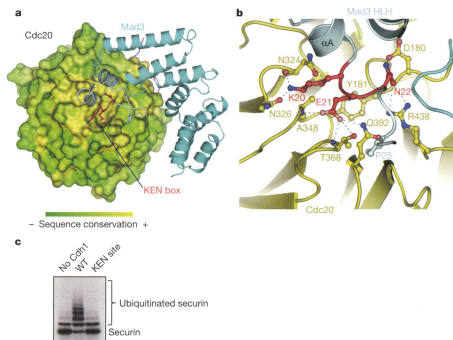


Figure 3 | The KEN box binds to a conserved surface at the centre of the top side of the WD40 domain. **a**, Cdc20 shown with surface representation coloured according to the sequence conservation of Cdc20 and Cdh1 (yellow (invariant) to green (less conserved)) (Supplementary Fig. 5). Mad3 diagram with KEN box in red. **b**, Details of KEN-box interactions with the Cdc20 WD40 domain. **c**, Mutation of the KEN-box-binding site abolishes *S. cerevisiae* APC/C^{Cdh1}-mediated ubiquitination of securin as indicated by an *in vitro* transcription or translation (IVT)-based ubiquitination assay. KEN-box mutants: N405A, N407A, Q473A, R517L (*S. pombe* Cdc20: N324, N326, Q392, R438).

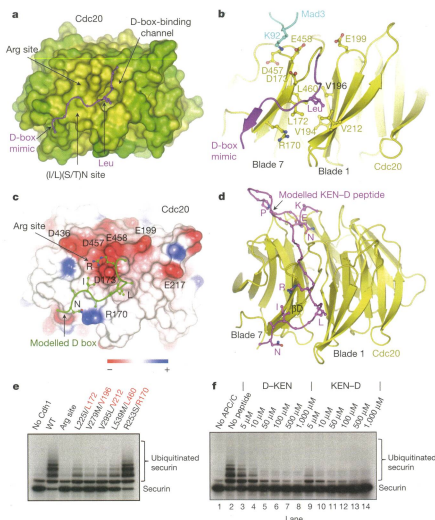


Figure 4 | The D box binds in an extended conformation to a conserved inter-blade channel on the Cdc20 WD40 domain. **a**, Cdc20 shown with surface representation coloured according to sequence conservation of Cdc20 and Cdh1 (yellow (invariant) to green (less conserved)). (Supplementary Fig. 5). **b**, D-box mimic bound through a peptide-in-channel mechanism to Cdc20. Lys 92 of Mad3 contacts the negatively charged D-box Arg-binding site of Cdc20, sterically hindering the D-box receptor of Cdc20. **c**, Negative electrostatic potential of Cdc20 in the vicinity of the Arg-binding site of modelled D box. **d**, Model of a KEN-D peptide bound to KEN and D-box sites of Cdc20. **e**, Mutation of the D-box-binding site abolishes *S. cerevisiae* APC/C^{Cdh1}-mediated ubiquitination of securin as indicated by an IUT-based ubiquitination assay. D-box Arg-binding-site mutants: D256S, D536S, E537S (*S. pombe* Cdc20: D173, D457, E458). Mutated *S. cerevisiae* Cdh1 residues are indicated in black with equivalent residues of *S. pombe* in red. At the D-box Leu-binding site, mutation of *S. pombe* Cdc20 Val 196 had the most notable effect, with little loss of activity for Leu460Met. Mutation of Arg 170, located distal to the Arg and Leu sites (Fig. 4c), did not affect APC/C^{Cdh1} activity. **f**, Ubiquitination competition assay. The KEN-D box peptide inhibits *S. cerevisiae* APC/C^{Cdh1}-mediated securin ubiquitination fivefold more efficiently than does the D-box-KEN-box peptide (compare lanes 5 and 10). Blocking the Leu-binding pocket by small molecules ligands may be a potential mechanism to inhibit APC/C-co-activator activity.

equivalents of the Cdc20 residues Asn 326, Thr 368, Gln 392 and Arg 438 in *Saccharomyces cerevisiae* Cdh1 abolished APC/C^{Cdh1}-catalysed ubiquitination of the KEN-box and D-box-dependent APC/C substrates securin and Clb2 (Fig. 3c and Supplementary Fig. 6). Co-migration of APC/C and Cdh1 on a native gel confirmed that the inactivation was not due to misfolding of the mutant co-activator (Supplementary Fig. 7). Our findings that the KEN box of Mad3 is embedded within a segment of ordered structure, possibly stabilized on the Mad3 HLH motif through contacts to Mad2 (Fig. 1), suggest that degrons do not need to be disordered to allow substrate recognition by ubiquitin ligases.

The WD40 domain of co-activators interacts with the D-box degnon (RXRLXX(I/L)(S/T/N)^{25,39}, acting as a co-receptor with the APC/C subunit Apc10 (refs 40, 41). However, the molecular details of co-activator-D box interactions have not been established. In the crystal structure of the MCC, an unexpected crystal packing contact, in which the C terminus of a neighbouring Mad3 subunit partially mimics a D box and contacts Cdc20, provides detailed molecular insights of co-activator-D box recognition. The D box mimic of Mad3 was found in an extended conformation engaged within a conserved channel located between blades 1 and 7 on the rim of the β -propeller toroid (Fig. 4b and Supplementary Fig. 8). The interaction is dominated by the burial of the aliphatic side chain of Leu 215 of Mad3, mimicking the Leu of the D-box RXRL motif, within a deep pocket perfectly matching a Leu side chain, and created by non-polar residues invariant in Cdc20 and Cdh1 (Fig. 4a and Supplementary Fig. 5). Other consensus residues of the D box—the essential and

invariant Arg and less-well-conserved (I/L)(S/T/N) motif⁴²—are not represented in the Mad3 D-box mimic. However, a candidate recognition site for the Arg side chain of the D box can be assigned to a cluster of negatively charged residues (Asp 173, Asp 457 and Glu 458) located on two adjacent β -strands of blade 7 on the top side of the D-box-binding channel. These residues are suitably positioned to interact with an Arg residue that is N-terminal to the anchored Leu residue, and are conserved in Cdh1 (Fig. 4b, c and Supplementary Fig. 5).

To test whether the putative D-box-binding site that was identified on Cdc20 is responsible for recognizing the D box of APC/C substrates, we generated mutants to abolish individually the equivalent Arg- and Leu-binding sites in Cdh1. Disrupting the Leu-binding pocket by replacing Val 196 with a bulky methionine, and substituting Ser for the Cdh1 equivalents of Asp 173, Asp 457 and Glu 458 at the putative Arg-binding site, eliminated the ability of Cdh1 to stimulate APC/C activity (Fig. 4e and Supplementary Fig. 6) but had no effect on co-activator binding to the APC/C (Supplementary Fig. 7). These results are consistent with the inter-blade channel of Cdc20 functioning as the D-box-binding site. The region of this channel that probably contacts the D-box (I/L)(S/T/N) motif is less well conserved between Cdc20 and Cdh1 (Fig. 4a), possibly explaining the differential affinity of the two co-activators for D-box degrons.

Substrate and inhibitor recognition by co-activators

To characterize further the APC/C degnon-binding sites on co-activators, we designed peptides that incorporate both KEN-box and D-box motifs and tested their ability to inhibit APC/C^{Cdh1}.

Examination of the Cdc20 structure suggested that a peptide with KEN and D boxes linked by 17 residues (KEN-D peptide) would enable cooperative binding of both degrons to their respective binding sites, thereby conferring higher-affinity binding than peptides with either degron alone (Fig. 4d). In contrast, a peptide with the reverse orientation of KEN and D boxes (D-KEN peptide), also connected by a 17-residue linker, would only permit a single degron to bind. When we compared the potential of these peptides to competitively inhibit securin ubiquitination by APC/C^{Cdh1}, we found that the KEN-D peptide was five times more potent as an inhibitor than the D-KEN peptide (Fig. 4f). Because D-KEN peptide inhibited APC/C^{Cdh1} with the same efficiency as a D-box peptide (Supplementary Fig. 9a), these results indicate that cooperative degron binding to Cdh1 was conferred by the specific spatial arrangement of the KEN and D boxes of the KEN-D peptide, in agreement with our assignment of the KEN- and D-box-binding sites on co-activators.

The spatial arrangement of KEN and D boxes in the KEN-D inhibitory peptide is markedly similar to the organization of KEN and D boxes in two APC/C inhibitors: Acml1 and Mesl1 (refs 43–45) (Supplementary Fig. 10). Both proteins inhibit the APC/C-co-activators through a pseudosubstrate-based mechanism that is dependent on their D and KEN boxes, and it seems reasonable to assume that the spacing of 18 and 24 residues between the KEN and D box degrons of Acml1 and Mesl1, respectively, optimizes inhibitor-co-activator affinity. Here we show one mechanism by which a KEN- and D-box-containing protein would bind co-activators. However, because of the diverse configuration and relative separation of KEN- and D-box motifs in APC/C substrates, several modes of co-activator-substrate recognition probably exist. Our assignment of the D-box recognition site to an inter-blade channel of the co-activator β -propeller was recently confirmed by a Cdh1–Acml1 crystal structure (W.C.H.C., D.B. and J. He, unpublished observations).

KEN- and D-box motifs adopt different conformations when bound to Cdc20; the KEN box is an underground helix (Fig. 3b), whereas the D box assumes an extended structure (Fig. 4b). This important distinction means that the three residues of the KEN motif are presented to the same surface of Cdc20, whereas in the D box alternative amino acid side chains are oriented in opposite directions. Thus, with the Leu side chain of the D box anchored by the co-activator, conserved Arg, Ile/Leu and Asn side chains at positions 1, 7 and 9 of the D box⁴², respectively, would be accessible to generate a composite D-box-co-activator recognition surface for the D-box co-receptor Apc10 (refs 40, 41). This is consistent with the identification of the D-box co-receptor at the interface of co-activator and Apc10 in an APC/C^{Cdh1-D box} ternary complex⁴¹.

Implications for MCC-mediated inhibition of the APC/C

The processes underlying MCC-mediated inhibition of APC/C are incompletely defined, and probably involve several mechanisms. Mad3, which is dependent on its N-terminal KEN box, blocks Cdc20-mediated substrate recognition¹⁵, consistent with our structure showing that the Mad3 KEN box binds to the KEN-box recognition site of Cdc20. Notably, the same KEN box also promotes Mad3-dependent APC/C-mediated degradation of Cdc20 (refs 18, 27, 29), which suggests that Mad3 has a role in positioning Cdc20 for ubiquitination by the APC/C's catalytic centre. To understand this function of Mad3, we docked our MCC coordinates into the electron-microscope-derived molecular envelope of the APC/C–MCC complex¹⁴ (Fig. 5). Interpretation of this structure was based on our previous subunit assignment and pseudo-atomic model of budding yeast APC/C^{Cdh1-D box} (ref. 46). The MCC crystal structure corresponds closely to the assigned MCC density of APC/C^{MCC}, with Cdc20, Mad2 and Mad3 clearly recognizable. Mad2 contacts the TPR subunits Cdc23 and Apc5, whereas Mad3 interacts with Apc1. There is insufficient unassigned density to account for the C-terminal kinase domain of BubR1 and Bub3, suggesting their structural disorder. Notably, the

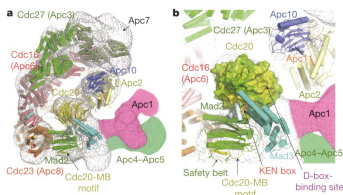


Figure 5 Pseudo-atomic structure of human APC/C^{MCC}. **a**, Docking of MCC coordinates into a human APC/C^{MCC} electron microscope map¹⁴. APC/C subunit coordinates are based on the pseudo-atomic structure of *S. cerevisiae* APC/C⁹. In the context of the APC/C^{MCC}, Cdc20 is in a downwards position relative to APC/C^{Cdh1} (ref. 41). **b**, Details of MCC interactions with APC/C showing the position of the D-box co-receptor on Cdc20 relative to Apc10 and the catalytic centre of Apc2 and Apc11. The downwards position of Cdc20 prevents interaction with Apc10. Surface representation of Cdc20 is colour-coded from yellow (invariant) to green (less conserved).

KEN-box-binding site of Cdc20 is blocked by Mad3, whereas the D-box site is directed towards, but not in contact with, its co-receptor Apc10 (Fig. 5b). Compared with APC/C^{Cdh1-D box}, in the APC/C^{MCC} complex, Cdc20 is displaced downwards towards Apc5. This position may facilitate Cdc20 ubiquitination. Furthermore, the lower position of co-activator prevents its D-box-binding site from generating a bipartite D-box co-receptor with Apc10 (ref. 41). This, together with a partial steric blockade of the arginine site of the D-box co-receptor of Cdc20 by Lys 92 of the Mad3 TPR domain (Fig. 4b), explains the inability of APC/C^{MCC} to recognize and ubiquitinate D-box-dependent substrates¹⁴. In APC/C^{MCC}, a region of the Cdc20 D-box-binding site for the Leu anchor and C-terminal residues, is accessible (Fig. 5b), suggesting that a non-consensus D-box sequence (that is, lacking an Arg residue and not dependent on the co-receptor Apc10) could engage this site.

In addition to the interactions of Mad2 and Mad3 with APC/C subunits, the position and activity of Cdc20 in the context of APC/C^{MCC} might also be influenced by the sequestration of its N-terminal APC/C recognition motifs by Mad2. The Mad2-binding motif of Cdc20 also mediates APC/C interactions¹⁷, which would be blocked by the Mad2 safety belt (Fig. 5b). Furthermore, constraining the Cdc20 N terminus by Mad2 might also prevent the neighbouring C box from accessing its APC/C-binding site, which is necessary for Cdc20-dependent stimulation of APC/C catalytic activity⁴⁸.

The SAC is antagonized by p31^{comet} (refs 30,49), and the structure of the C-Mad2–p31^{comet} complex showed that p31^{comet} binds at the Mad2 dimerization interface to inhibit the conformational activation of O-Mad2 (ref. 31). However, it is clear from our MCC structure that because Mad3 and p31^{comet} bind to a common Mad2 interface, p31^{comet} would compete for Mad3 interactions with Mad2 (Fig. 2c), explaining how p31^{comet} both antagonizes the assembly of the MCC³¹ and promotes its disassembly^{32,50}.

Here we show how the molecular basis for APC/C inhibition by its regulators is coupled to degron recognition by co-activators. APC/C activity is modulated by sterically blocking substrate recognition and through conformational changes that disrupt the substrate-binding site, reminiscent of modes of protein kinase regulation. This contrasts with the SCF complex whose activity is regulated at the level of substrate recognition through degron phosphorylation.

METHODS SUMMARY

Expression, purification and crystallization of *S. pombe* MCC. The *S. pombe* MCC (involving Cdc20, Mad2 and Mad3) was generated by co-expressing

Cdc20-Slp1 (residues 87–488), Mad2 (with Leu12Ala and Arg133Ala mutations) and Mad2 (residues 1–223) in the baculovirus and insect cell systems. Crystallization, crystal structure determination, mutagenesis, enzyme assays and other procedures were performed as described in the Methods. Diffraction data were collected at beamline I02 at the Diamond Light Source.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions W.C.H. cloned, purified and crystallized the *S. pombe* MCC, and performed the biochemistry and mutagenesis experiments. W.C.H., K.K. and D.B. collected the X-ray diffraction data. K.K. determined the complex structure and modelled the D-box and KEN-D peptides. Z.Z. advised on cloning strategies of the MCC. E.H.K. provided purified endogenous APC/C, W.C.H., K.H.K. and D.B. docked crystal coordinates into the human APC^{MCC} electron-microscope map. W.C.H. and D.B. wrote the manuscript and the others authors provided editorial input.

Author Information The MCC coordinates have been deposited in the Protein Data Bank under accession number 4aez. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to D.B. (david.barford@icr.ac.uk).

Structural basis of ultraviolet-B perception by UVR8

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The *Arabidopsis thaliana* protein UVR8 is a photoreceptor for ultraviolet-B. Upon ultraviolet-B irradiation, UVR8 undergoes an immediate switch from homodimer to monomer, which triggers a signalling pathway for ultraviolet protection. The mechanism by which UVR8 senses ultraviolet-B remains largely unknown. Here we report the crystal structure of UVR8 at 1.8 Å resolution, revealing a symmetric homodimer of seven-bladed β -propeller that is devoid of any external cofactor as the chromophore. Arginine residues that stabilize the homodimeric interface, principally Arg 286 and Arg 338, make elaborate intramolecular cation- π interactions with surrounding tryptophan amino acids. Two of these tryptophans, Trp 285 and Trp 233, collectively serve as the ultraviolet-B chromophore. Our structural and biochemical analyses identify the molecular mechanism for UVR8-mediated ultraviolet-B perception, in which ultraviolet-B radiation results in destabilization of the intramolecular cation- π interactions, causing disruption of the critical intermolecular hydrogen bonds mediated by Arg 286 and Arg 338 and subsequent dissociation of the UVR8 homodimer.

Perception of light is important for all kingdoms of life¹. Light regulates the circadian clock in worms and social activity in fruitflies^{2,3}. In plants, light is a major source of energy and regulates all major developmental and physiological processes^{4,5}. A wide wavelength range of light is sensed by specific families of photoreceptors: phytochrome for red and far red⁶; phototropin and cryptochrome for ultraviolet-A and blue⁷⁻¹¹; and UVR8 for ultraviolet-B (wavelength range 280–315 nm)^{4,12}. Except for UVR8, all other photoreceptors contain specific external cofactors as chromophores: bilin for phytochrome; FAD and MTHF for cryptochrome; and FMN for phototropin^{4,13}. It remains unclear whether UVR8 contains any external cofactor for ultraviolet-B perception.

UVR8, originally identified as a regulatory protein for ultraviolet-B-triggered signal transduction¹⁴, was recently shown to be a receptor for ultraviolet-B¹⁵. Ultraviolet-B perception was thought to induce dissociation of the UVR8 homodimer, allowing its subsequent interaction with COP1 and transcriptional activation of ultraviolet-B-responsive genes^{2,15-17} (Fig. 1a). A number of tryptophan residues, particularly Trp 285, were shown to have an important role in ultraviolet-B-triggered signalling¹². Despite these advances, it remains unknown how ultraviolet-B is sensed by UVR8 or how ultraviolet-B perception leads to dissociation of the UVR8 homodimers.

Biochemical characterization of UVR8

The full-length, wild-type UVR8 and two variants, W285F and W285A, were purified to homogeneity. As reported¹², both wild type and W285F existed mainly as homodimers on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the absence of heating (Fig. 1b, lanes 1–2). However, only wild type, not W285F, was able to undergo ultraviolet-B-induced monomerization (lanes 7–8). By contrast, the variant W285A appeared only as a monomer both before and after ultraviolet-B irradiation (lanes 3 and 9). Heating at 96 °C in the presence of SDS reduced all UVR8 homodimers to a monomeric state (lanes 4–6). These results are in agreement with published observations¹².

The ionic detergent SDS is a protein denaturant. Homodimer formation of wild-type UVR8, however, is remarkably stable and resists treatment by up to 12% SDS in the absence of heating (Supplementary Fig. 1a). Because the SDS sample buffer contains 200 mM dithiothreitol (DTT), we speculated that two molecules of UVR8 are held together through a covalent linkage—such as a disulphide bond—that is susceptible to heating in the presence of DTT or ultraviolet-B irradiation. Quite unexpectedly, elevated ionic strength led to efficient conversion of the UVR8 homodimers to a monomeric state (Supplementary Fig. 1b). This observation strongly suggests that the forces that hold together two UVR8 molecules are ionic in nature.

Use of SDS-PAGE may not allow faithful evaluation of native protein conformation. To alleviate this potential problem, we used the more sensitive method of gel filtration to examine the oligomeric state of UVR8 under physiological pH and ionic strength (Fig. 1c). Before ultraviolet-B irradiation, wild-type UVR8 was eluted from gel filtration with an apparent molecular mass of approximately 150 kDa, larger than that calculated for a UVR8 homodimer (~100 kDa). This discrepancy is probably caused by the extended flexible sequences at the amino and carboxy termini of UVR8. After ultraviolet-B irradiation, the elution volume for wild-type UVR8 corresponded to a molecular mass of about 75 kDa (Fig. 1c). This observation confirms the reported, ultraviolet-B-induced dimer-to-monomer switch¹². As anticipated, the variant UVR8(W285F) appeared as a homodimer irrespective of ultraviolet-B radiation (Fig. 1c). However, unlike the SDS-PAGE result¹² (Fig. 1b), UVR8(W285A) existed mainly as a homodimer both with and without ultraviolet-B treatment (Fig. 1c). Ultraviolet-B irradiation seemed to have weakened formation of the W285A homodimer as judged by the presence of a small fraction of monomers (Fig. 1c).

The WD40 repeats of UVR8 are thought to be responsible for ultraviolet-B perception¹². Supporting this notion, the protease-resistant core domain of UVR8 (residues 12–381; Supplementary

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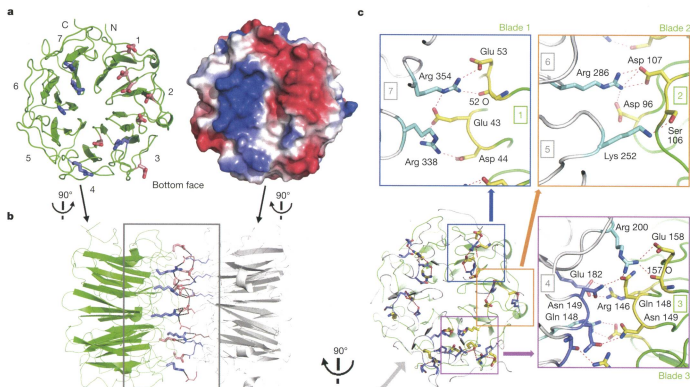


Figure 2 | Structural basis of UVR8 homodimer formation. **a**, The bottom face of the UVR8 propeller contains two surface patches of complementary charges. The left and right panels depict the cartoon and electrostatic surface representations of the UVR8 core domain, respectively. Five acidic amino acids (coloured red) from blades 1 and 2 constitute the negatively charged patch, whereas four basic residues (coloured blue) form the positively charged patch.

rings of Trp 302 and Trp 285, respectively, allowing cation– π interactions of maximal strength²². Arg 286 also interacts with the phenyl ring of Tyr 253 and weakly associates with Trp 250. In addition, the indole ring of Trp 285 associates with the guanidinium group of Arg 338 through strong cation– π interactions, whereas the indole ring of Trp 285 binds to Trp 233 and Trp 337 through π – π stacking interactions²⁴ (Fig. 3b). The essence of π – π stacking is the cation– π interaction, as the edge of the tryptophan indole ring (around the amine group) carries net positive charges²³. Furthermore, Trp 233 appears to have an important role in this network by making π – π stacking interactions with Trp 337 and strong cation– π interactions with Arg 234 (Fig. 3b, c). It is unusual to have such a high density of cation– π interactions within a protein structure²¹ (Fig. 3c).

Ultraviolet-B chromophore identification

Because UVR8 contains no external cofactor, the chromophore for ultraviolet-B perception must be amino acid(s). Among the 20 naturally occurring amino acids, only tryptophan and tyrosine, with maximal absorption wavelengths of 280 and 275 nm, respectively, are potentially capable of perceiving ultraviolet-B (280–315 nm). The UVR8 core domain contains thirteen tryptophan and eight tyrosine residues. Six of the thirteen tryptophans are located in the hydrophobic core and away from the homodimeric interface (Supplementary Fig. 7), making them unlikely candidates for ultraviolet-B perception. Among the eight tyrosine residues, only Tyr 253 is located close to the homodimeric interface (Fig. 3b). This analysis suggests that the chromophore for ultraviolet-B is among the seven tryptophan residues at the homodimeric interface.

Ultraviolet-B perception is probably coupled with chemical and/or conformational changes around the chromophore, presumably leading to alteration of its fluorescence emission spectra. Mutation of the

b, Formation of UVR8 homodimer is mediated by charge-stabilized hydrogen bonds, mainly from the two surface patches of complementary charges. The two UVR8 molecules are related to those in panel **a** by 90° rotations, as indicated in the figure. **c**, A close-up view on the intermolecular hydrogen bonds at the homodimeric interface. Hydrogen bonds are represented by red dashed lines.

key chromophore should result in abrogation of its ability to sense ultraviolet-B and consequent loss of fluorescence changes that are characteristic of ultraviolet-B perception. To identify conclusively the chromophore, we individually mutated the seven tryptophan residues to phenylalanine.

First, we examined the intrinsic tryptophan fluorescence emission spectra for wild-type UVR8. In the absence of prior ultraviolet-B treatment, wild-type UVR8 was continuously monitored at an emission wavelength of 335 nm, with an ultraviolet-B excitation wavelength of 295 nm. Consistent with saturable ultraviolet-B perception, the fluorescence signal increased rapidly over the initial 200 s and reached a maximum after 400 s (Fig. 4a, top). Prolonged irradiation, however, resulted in a gradual decrease of the fluorescence signal (Supplementary Fig. 8a), presumably due to fluorescence quenching. With prior ultraviolet-B treatment, the fluorescence signal of wild-type UVR8 decreased very slowly over time (Fig. 4a, top), again due to fluorescence quenching. By sharp contrast, the UVR8 variants W285F and W285A completely lost the ability to perceive ultraviolet-B (Fig. 4a, bottom; Supplementary Fig. 8b). These observations identify Trp 285 as an essential component of the chromophore.

Next, we examined the other six UVR8 missense proteins. Most notably, the variant W233F no longer showed an increase in fluorescence induced by ultraviolet-B (Fig. 4b, left), suggesting that Trp 233 also has an essential role in ultraviolet-B perception. By sharp contrast, each of the five variants W337F, W302F, W250F, W198F and W94F retained the ability to sense ultraviolet-B and to undergo ultraviolet-B-induced dimer-to-monomer switches (Fig. 4b and Supplementary Fig. 8c; W94F, data not shown). These analyses unambiguously identify Trp 285 and Trp 233 collectively as the chromophore for ultraviolet-B perception. Consistent with this conclusion, Trp 285 and Trp 233 are involved in considerably more and stronger cation– π

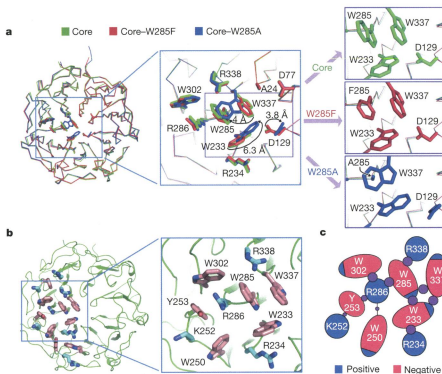


Figure 3 | Structural features of the ultraviolet-B-sensing amino acids.

a, Structural comparison of UVR8 core domain (coloured green) with the variants W285F (red) and W285A (blue). **b**, Significant conformational changes occur to Trp 337, Trp 233 and Asp 129. **c**, A close-up view on the putative ultraviolet-B-sensing residues. Arginine and tryptophan residues are differentially coloured. **c**, A schematic diagram of the extensive network of

interactions compared to other tryptophans such as Trp 337 and Trp 198 (Supplementary Fig. 9).

Ultraviolet-B perception by Trp 285 and Trp 233 probably results in disruption of the cation- π interactions with key arginine residues, causing disruption of arginine-mediated intermolecular hydrogen bonds and consequent dissociation of UVR8 homodimers. To identify the key arginine residues, we generated five UVR8 variants, each targeting an arginine at the UVR8 homodimeric interface. Compared to wild-type UVR8, the variants R286A and R338A exhibited a grossly altered ability to perceive ultraviolet-B (Supplementary Fig. 10, left). Notably, these two UVR8 variants existed exclusively in a monomeric state (Supplementary Fig. 10, right), consistent with the important role of Arg 286 and Arg 338 at the homodimeric interface. By contrast, the variants R354A, R200A and R146A retained the abilities to perceive ultraviolet-B and to undergo an ultraviolet-B-induced dimer-to-monomer switch (Supplementary Fig. 10).

Transient nature of monomeric UVR8

Absorption of ultraviolet-B is predicted to excite the indole rings of the chromophore Trp 285 and Trp 233. Because the excited indole rings may dissipate energy to return to their ground state, we suspected that the ultraviolet-B-irradiated UVR8 monomers may revert back to homodimers over time. To examine this prediction, we subjected the UVR8 core domain to a saturating amount of ultraviolet-B radiation, left the protein at room temperature (23 °C) to recover in the absence of ultraviolet-B, and applied aliquots of the protein to SDS-PAGE at various time points. The result clearly shows that the UVR8 monomer slowly but steadily converted back to the homodimeric state (Fig. 4c). The partially recovered UVR8 homodimers can be completely monomerized again by ultraviolet-B irradiation (Fig. 4c, lane 8). The transient nature of monomeric UVR8 has obstructed repeated crystallization efforts, which were designed to capture the conformation of the ultraviolet-B-activated UVR8 core domain. The crystals were eventually

obtained but only contained the homodimeric form (Fig. 4d). Obviously, ultraviolet-B-irradiated UVR8 core domain in the crystallization drops slowly reverted to a dimeric state before crystallization. Nonetheless, we solved this structure, which is identical to that of the UVR8 core domain before ultraviolet-B irradiation.

Perspective

Photoreceptors rely on chromophores to perceive light. For example, phytochrome covalently associates with a single bilin molecule^{25,26}, cryptochrome contains a non-covalently-bound FAD molecule^{27,28}, and phototropin depends on FMN^{29,30}. In contrast to all known photoreceptors, UVR8 does not contain any external cofactor and instead uses two tryptophan residues, Trp 285 and Trp 233, as the chromophore for ultraviolet-B perception. This seems natural, because the absorption wavelengths for tryptophan coincide with the range of ultraviolet-B. Consequently, the ultraviolet-B-sensing mechanism of UVR8 differs markedly from those of other photoreceptors.

Our experimental evidence, in conjunction with knowledge of tryptophan fluorescence, yields a mechanistic model of ultraviolet-B perception by UVR8 (Fig. 4e). Ultraviolet-B irradiation results in excitation of the Trp 285 and Trp 233 indole rings, which is thought to disrupt the π -bond over the indole rings, leading to destabilization and abrogation of the intramolecular cation- π interactions. Such disruption is likely to trigger conformational switch of the side chains of Arg 286 and Arg 338, which would no longer be able to maintain intermolecular hydrogen bonds with Asp or Glu residues from the neighbouring UVR8 molecule, causing dissociation of the UVR8 homodimer (Fig. 4e). Furthermore, the excited indole rings are known to undergo a process of excited-state proton transfer³¹, which allows the indole ring to carry a positive charge and completely destroys the cation- π interactions. Importantly, excited-state proton transfer also leads to quenching of intrinsic tryptophan fluorescence, providing a plausible explanation for the observed slow decrease of

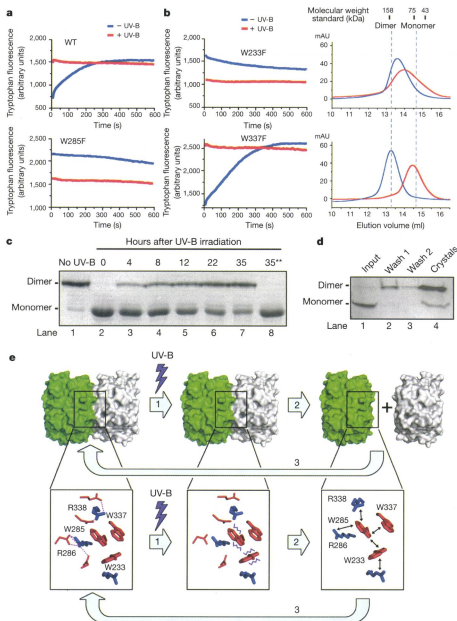


Figure 4 | Identification of Trp 285 and Trp 233 as the ultraviolet-B chromophore. **a**, Identification of Trp 285 as an essential ultraviolet-sensing amino acid. In the absence of prior ultraviolet-B (UV-B) radiation, the wild-type (WT) UVR8, but not the variant W285A, displayed a temporal, ultraviolet-B-induced increase of tryptophan fluorescence. This fluorescence increase completely disappeared after pre-irradiation by ultraviolet-B. **b**, The mutation W233F, but not W337F, in UVR8 led to loss of ultraviolet responsiveness as judged by tryptophan fluorescence (left panels). The variant W233F is no longer a stable homodimer, both with and without ultraviolet-B irradiation (top right). By contrast, the variant W337F can still undergo dimer-to-monomer switch in response to ultraviolet-B treatment (bottom right). **c**, Ultraviolet-B-irradiated UVR8 core domain slowly reverted back to a homodimeric state as judged by SDS-PAGE. Notably, 35 h after ultraviolet-B

irradiation, the partially recovered UVR8 homodimers were completely monomerized again by ultraviolet-B irradiation (lane 8, indicated by double asterisk). **d**, The crystals derived from ultraviolet-B-irradiated UVR8 core domain mostly contained the homodimeric form. Crystals were washed twice with crystallization buffer and examined by SDS-PAGE. **e**, A proposed mechanism for ultraviolet-B sensing by UVR8. In step 1, ultraviolet-B radiation excites Trp 285 and Trp 233. The excited states (purple wavy lines) of Trp 285 and Trp 233 are unable to maintain the cation- π interactions with surrounding residues. In step 2, disruption of the intramolecular cation- π interactions results in pronounced changes of side-chain conformations (black arrows), disrupting the intermolecular hydrogen bonds and causing dissociation of UVR8 homodimers. In step 3, Trp 285 and Trp 233 dissipate energy to return to the ground state, which allows re-formation of homodimers.

fluorescence signal. Notably, Asp 129, Glu 182 and Arg 234 are all located in close proximity to Trp 233 and Trp 285 (Supplementary Fig. 11) and may well serve as proton donors. In this model, ultraviolet-B perception involves no covalent modification of UVR8, such as tryptophan oxidation or crosslinking. This notion is supported by mass spectrometric analysis (Supplementary Fig. 12). Further supporting this conclusion, ultraviolet-B-induced monomerization of UVR8 was unaffected by the presence of strong reducing or oxidizing agents (Supplementary Fig. 13).

The observed fluorescence emission represents the total input from all 13 tryptophan residues and potentially other aromatic amino acids in UVR8. However, only those residues that are affected by ultraviolet-B perception or ultraviolet-B-induced environmental changes contribute to alteration of the fluorescence signal. The fact that a missense mutation of either Trp 285 or Trp 233 completely abrogates increase of the fluorescence signal identifies these two amino acids collectively as the chromophore for ultraviolet-B. In addition, this finding also strongly suggests that other tryptophan residues in UVR8 do not undergo any

significant ultraviolet-B-induced environmental changes. This analysis also validates the measurement of intrinsic tryptophan fluorescence as a sensitive method for the detection of ultraviolet-B perception.

Our study serves as a framework for understanding mutant phenotypes in plants. For example, the *Arabidopsis* mutants G1455 and G202R lost the ability to perceive ultraviolet-B and the mutant proteins G1455 and G202R were no longer able to form a homodimer¹². In our crystal structure, Arg 146 and Arg 200 make an important contribution to stabilize formation of the UVR8 homodimer (Fig. 2c); both Gly 145 and Gly 202 are located in close proximity to Arg 146 and Arg 200, respectively (Supplementary Fig. 14). Thus the mutations G1455 and G202R may have a deleterious consequence on homodimer formation.

Despite revealing the structure revelation and underlying mechanism, important questions remain about the UVR8-mediated signalling pathway. It is unclear how UVR8 interacts with the central regulator of light signalling, COP1. However, the UVR8-COP1 complex regulates downstream signalling events is uncertain. Answers to these questions await further experimental investigations.

While this manuscript was under final revision at *Nature*, Christie *et al.* reported similar findings in *Science*²¹.

METHODS SUMMARY

Wild-type UVR8 and all variants were subcloned by standard molecular biology, expressed, and purified. Tryptophan fluorescence was measured in a fluorescence spectrophotometer (Hitachi F-4600), with excitation and emission wavelengths of 295 and 335 nm, respectively. Gel filtration was used to observe ultraviolet-B-induced conformational changes. All crystals were generated by the hanging-drop vapour-diffusion method. All data sets were collected at the Shanghai Synchrotron Radiation Facility (SSRF) beamline BL17U and the Spring-8 beamline BL41XU and processed using the HKL2000 package²². Further processing was carried out using programs from the CCP4 suite²³. The UVR8 structure was solved using the SeMet-derived UVR8(W285A) crystals. The selenium positions were determined using the program SHELXD²⁴. A partial model was traced automatically using the program BUCCANEER²⁵. The resulting map was in good quality and the partial model was manually rebuilt in COOT²⁶. Sequence assignment was aided with the selenium sites in the anomalous difference Fourier map. The final structure was refined with PHENIX²⁷. Using UVR8(W285A) coordinates as the initial search model, crystal structures of W285F and wild-type UVR8 were solved by molecular replacement using PHASER²⁸ and refined with COOT²⁶ and PHENIX²⁸.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Information The atomic coordinates and structure factor files of UVR8 wild type, W285A and W285F have been deposited in the Protein Data Bank under accession codes 4DNW, 4DNU and 4DNU, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to Y.S. (shi-lab@tsinghua.edu.cn).

A close halo of large transparent grains around extreme red giant stars

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An intermediate-mass star ends its life by ejecting the bulk of its envelope in a slow, dense wind^{1–3}. Stellar pulsations are thought to elevate gas to an altitude cool enough for the condensation of dust¹, which is then accelerated by radiation pressure, entraining the gas and driving the wind^{2,4,5}. Explaining the amount of mass loss, however, has been a problem because of the difficulty of observing tenuous gas and dust only tens of millarcseconds from the star. For this reason, there is no consensus on the way sufficient momentum is transferred from the light from the star to the outflow. Here we report spatially resolved, multiwavelength observations of circumstellar dust shells of three stars on the asymptotic giant branch of the Hertzsprung–Russell diagram. When imaged in scattered light, dust shells were found at remarkably small radii (less than about two stellar radii) and with unexpectedly large grains (about 300 nanometres in radius). This proximity to the photosphere argues for dust species that are transparent to the light from the star and, therefore, resistant to sublimation by the intense radiation field. Although transparency usually implies insufficient radiative pressure to drive a wind^{6,7}, the radiation field can accelerate these large grains through photon scattering rather

than absorption⁸—a plausible mass loss mechanism for lower-amplitude pulsating stars.

We observed W Hydrae, R Doradus and R Leonis using aperture-masked^{9,10} polarimetric interferometry (Fig. 1), along with dust-free stars to verify our detection methodology. Figure 1 shows the ratio of the horizontally and vertically polarized visibilities ($V_{\text{horiz}}/V_{\text{vert}}$), plotted as a function of baseline azimuth and length. The dust-free star 2 Centauri, which has no polarized flux, shows a constant ratio $V_{\text{horiz}}/V_{\text{vert}} = 1.0$ within its uncertainties. However, the dust-enshrouded star W Hya shows a strong sinusoidal variation of $V_{\text{horiz}}/V_{\text{vert}}$ with azimuth, as expected from a resolved circumstellar scattering shell. By taking advantage of spherical symmetry, we produced the much simpler baseline-dependent observable plotted in Fig. 2. A model was then fitted to the data to determine the dust shell radius and the amount of light scattered by the shell at each wavelength (Supplementary Information). These results are summarized in Table 1. Figure 3 shows the model image of the star and shell as seen in orthogonal polarizations for W Hya at a wavelength of 1.24 μm , from which the model visibilities were derived.

Scattered-light dust shells around the three asymptotic giant branch (AGB) stars observed were found close to the star, at radii $\lesssim 2$ stellar radii. This is in contrast to earlier models that place the shell at many stellar radii¹, but is consistent with some recent models⁶ and with interferometric¹¹ and polarimetric¹² measurements. On the basis of typical elemental abundances and spectral observations, the composition of

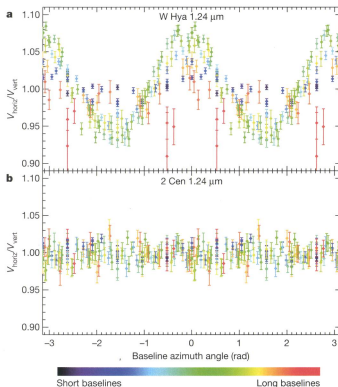


Figure 1 | Polarimetric interferometry of W Hya at 1.24 μm . Although light scattered by each part of the circumstellar dust shell is strongly polarized, the integrated signal recovered with conventional polarimetry is zero for an unresolved spherically symmetric shell. In this study, aperture-masking interferometry^{9,10} (which converts the 8-m pupil of the Very Large Telescope into a multi-element interferometer, using the NACO²⁴ instrument) allows access to the ~ 10 -mas spatial scales required to resolve the shell, and polarimetric measurements (obtained by simultaneously measuring interferometric visibilities in orthogonal polarizations²⁵) allows light from the star and light from the dust shell to be differentiated. Here the ratio of the horizontally polarized visibilities (V_{horiz}) to vertically polarized visibilities (V_{vert}) is plotted against baseline azimuth angle (corresponding to position angle on the sky). Colour encodes the baseline length (longest, 7.3 m; shortest, 0.56 m). The ratio $V_{\text{horiz}}/V_{\text{vert}}$ is a differential observable, which allows the cancellation of residual systematic errors and depends only on the fractional polarized scattered light signal. **a**, Result for W Hya, an AGB star with a circumstellar shell; $V_{\text{horiz}}/V_{\text{vert}}$ deviates from 1, varying sinusoidally with azimuth. This is the signal expected from a thin, spherically symmetric dust shell scattering the light from a central star. This signal varies in amplitude for different baselines, encoding the spatial extent of the resolved structure. Data points have been repeated over two cycles. The longest baselines (red) have poor signal-to-noise ratios because they are close to the null, where the visibility curve of the star is extremely low. Error, 1 σ . **b**, Visibility data for the star 2 Cen, which has no circumstellar dust shell and, hence, no polarized signal from scattering; here $V_{\text{horiz}}/V_{\text{vert}} \approx 1$ for all azimuths. Errors, 1 σ .

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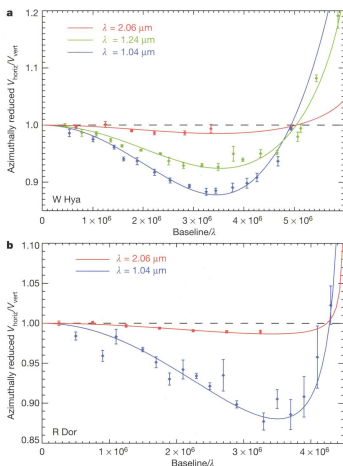


Figure 2 | Wavelength dependence of scattering for W Hya and R Dor. The azimuthally reduced ratio $V_{\text{horiz}}/V_{\text{vert}}$ for the AGB stars W Hya and R Dor, plotted against spatial frequency (baseline length divided by wavelength, λ), at multiple wavelengths. The functional form of the visibility ratio excursions of all three AGB stars are consistent, within uncertainties, with a simple spherically symmetric shell. We were therefore able to enhance our signal-to-noise ratio significantly in a quantitative analysis by reducing our two-dimensional visibility data to a one-dimensional function of the baseline length (corresponding to spatial frequency). This was achieved by dividing $V_{\text{horiz}}/V_{\text{vert}}$ by the expected sinusoidal variation (characteristic of a spherical shell) at a fixed amplitude, resulting in the much simpler, baseline-dependent observable plotted here. Points are the observed data (binned, errors, 1 σ) and the solid lines are the fitted model. The spatial frequency of the minimum of the characteristic 'dip' varies with the radius of the dust shell: for a larger shell, the minimum of the dip occurs at lower spatial frequencies. The depth of the dip depends on the amount of scattered light, with a larger deviation from $V_{\text{horiz}}/V_{\text{vert}} = 1.0$ indicating that a larger fraction of the total flux arises from light scattered by the shell. This is seen to decrease strongly at longer wavelengths as expected theoretically; the precise change in this quantity as a function of wavelength can be used to determine the dust grain radius using Mie scattering theory (Fig. 4). The fitted parameters for these quantities are included in Table 1. The 2.06- μm data have insufficient spatial resolution to constrain the shell size, so for models at these wavelengths we fixed the shell size to be consistent with the fitted size at shorter wavelengths.

possible solution to this dilemma arises when very large grains are considered.

The degree of scattering by dust depends strongly on the wavelength of the incident light and on the size of the particles. By analysing our multiwavelength measurements using Mie scattering theory, we determined the effective grain size and the number of grains. As shown in Fig. 4, we found an effective grain radius of ~ 300 nm. For grains of this size, the scattering opacity becomes very large, well beyond that resulting from Rayleigh scattering when the particles are smaller. In this regime, the contribution to radiative acceleration by scattering, rather than by absorption alone, must be considered. Models show that grains exceeding a certain critical scattering opacity can drive a wind at high magnesium condensation and that, for a star of temperature 2,700 K, this critical scattering opacity is only exceeded in a narrow range of dust grain radii around 300 nm (ref. 8). We also note that a narrow range of grain radii, of the order of ~ 500 nm, is predicted on the basis of a self-regulating feedback mechanism: grain growth effectively halts once the critical size is reached, because the dust is then accelerated outwards and gas densities quickly decrease⁸. This is consistent with observations of grains in the interstellar medium, which are dominated by silicates¹⁵ and have similar grain sizes^{16,17}. Wind driving due to scattering by magnesium-rich silicates is consistent with the finding that mass loss in AGB stars depends on their metallicity¹⁸. Although this model encounters difficulties in the case of stars with extremely extended atmospheres, such as R Leo (owing to the mass of the stellar atmosphere at and above the dust-forming layers being too high to allow sufficient acceleration¹⁹), it provides a plausible explanation for the mass loss of semiregular pulsating stars such as R Dor. Our

AGB dust shells is expected to be dominated by silicates^{8,13,14} in the form of olivine ($\text{Mg}_2\text{Fe}_{2(1-x)}\text{SiO}_4$) and/or pyroxene ($\text{Mg}_x\text{Fe}_{(1-x)}\text{SiO}_3$), where $0 \leq x \leq 1$. The temperature of a grain is determined by its opacity, that is, how strongly it absorbs the surrounding radiation field. Multiwavelength models⁸ show that silicate dust that contains iron absorbs the stellar flux strongly (as these dust species have high opacities at wavelengths of $\sim 1 \mu\text{m}$, where the energy distribution peaks) and so can only condense at distances greater than ~ 5 stellar radii. These iron-rich species could be accelerated by absorption of stellar radiation, but they form too far from the star to provide an efficient mass loss mechanism for low-amplitude pulsators⁶ (semiregular variable stars). Our detection of dust much closer to the star is instead consistent with the presence of iron-free silicates such as forsterite (Mg_2SiO_4) and enstatite (MgSiO_3), which are almost transparent at wavelengths of $\sim 1 \mu\text{m}$. Such grains do not heat to sublimation, despite the intense radiation close to the star, but the same transparency also prevents the momentum transfer from starlight required to drive a wind. For some stars, a

Table 1 | Summary of fitted model parameters

Star	Φ	λ (μm)	R_{star} (mas)	R_{shell} (mas)	Scattered fraction	Grain radius (nm)	Scattering-shell mass
R Dor	0.7	1.04	27.2 ± 0.2	43.3 ± 0.3	0.124 ± 0.003	299 ± 39	$(2.7 \pm 0.2) \times 10^{-10} M_{\odot}$
		2.06	27.7 ± 1.4	43.6 ± 3.2	0.014 ± 0.002		
W Hya	0.2	1.04	18.7 ± 0.4	37.9 ± 0.2	0.176 ± 0.002	316 ± 4	$(1.04 \pm 0.02) \times 10^{-8} M_{\odot}$
		1.24	18.9 ± 0.5	37.0 ± 0.3	0.110 ± 0.003		
		2.06	18.9 (fixed)	37.0 (fixed)	0.022 ± 0.004		
		1.04	16.3 ± 0.3	29.9 ± 0.4	0.120 ± 0.003		
R Leo	0.4	1.04				$\sim 300^*$	$\sim 2 \times 10^{-10} M_{\odot}$

The radii of the dust shells were found to be $\leq 2R_{\text{star}}$. The scattered fraction is the proportion of the total flux arising from scattering by the dust shell. The grain radius was obtained from fitting to multiwavelength observations using Mie scattering (with the value for R Leo fixed at 300 nm). The scattering-shell mass was calculated only for the observed dust (and not, for example, for a distribution extending to small grains invisible to our technique). Stellar radii given are for a uniform disc. All three AGB stars (W Hya, R Dor and R Leo) were observed in March 2009, with additional observations of W Hya at 1.24 μm and 2.06 μm made in June 2010. Although the photospheric diameter, and possibly the dust shell diameter, are expected to vary throughout the stellar pulsation cycle, the two sets of observations for W Hya were taken approximately one period apart and have therefore been combined. These figures assume both the dust to be iron-free silicate (forsterite) grains of uniform size and that to be full magnesium silicate. Full magnesium condensation is a reasonable assumption for the stars with more compact atmospheres (for example R Dor) but is inconsistent with observed optical depths for stars with more extended atmospheres¹⁹. In the event that there is also a population of small, weakly scattering grains that do not show up in our data, these values represent lower limits, with the total shell mass being greater. Furthermore, if the shell is geometrically extended then the true mass will be greater, as these calculations assume a thin shell. The uncertainties given are based on random errors and do not account for systematic errors such as those described here. Hipparcos parallaxes²⁰ and experimentally measured optical constants²¹ have been used. Φ indicates visual phase, derived from the AAVSO International Database; R_{star} , stellar radius; R_{shell} , radius of dust shell.

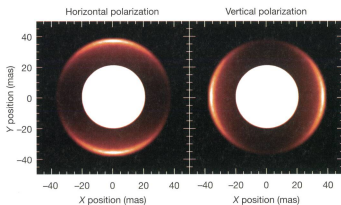


Figure 3 | Model image for W Hya with circumstellar shell viewed in horizontally and vertically polarized light. The white disc represents the uniform-disk star used in the model. A three-dimensional model of a star with a thin scattering shell was constructed, and the scattered intensity observed in each polarization for each point on the shell was calculated using Mie scattering, yielding an image of the star and shell. We then derived polarized visibilities from the model and fitted them to the observed visibilities, to determine the dust shell radius and the scattered fraction. Details of the modelling process can be found in Supplementary Information. See Supplementary Fig. 2 for a diagram illustrating how the polarized intensity distribution arises.

observations provide direct evidence for a population of dust grains capable of powering a scattering-driven wind.

The last column of Table 1 gives the mass of the dust that contributes to the observed scattering signal, assuming the shell to be thin and the dust grains to be forsterite of a uniform size. If full magnesium condensation and solar abundances are assumed, then the gas-to-dust ratio is ~ 600 , which yields total shell masses of $\sim 6 \times 10^{-7} M_{\odot}$, $\sim 2 \times 10^{-7} M_{\odot}$ and $\sim 1 \times 10^{-7} M_{\odot}$ for W Hya, R Dor and R Leo, respectively. Because the pulsation periods of these stars are ~ 1 yr and the mass loss rates are $\sim 1 \times 10^{-7} M_{\odot} \text{ yr}^{-1}$ (refs 20, 21), this implies that for stars with less extended atmospheres a large fraction of the observed shell is ejected each pulsation cycle, consistent with the observed dust being part of an outflow. In the extended-atmosphere case, where full magnesium condensation is not observationally supported, a possible alternative dust species is corundum (Al_2O_3).

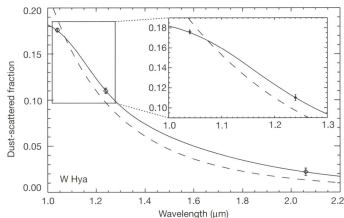


Figure 4 | Grain size measurement. Grain size fitted to the fraction of scattered light as a function of wavelength, for W Hya. Inset, magnified view of the 1.0–1.3-μm region. The solid line represents the fitted Mie scattering model (where grain size and grain number were fit parameters), and the dashed line represents the best Rayleigh fit (where grain size was fixed to be below the Rayleigh limit). The data are inconsistent with Rayleigh scattering. The fitted Mie model yields an effective grain radius of 316 ± 4 nm. In reality, a distribution of grain sizes may be present; for example, a population of very much smaller particles would contribute only weakly and could be undetected. Our data show that, regardless of the presence or absence of smaller grains, a population of large, ~ 300 -nm, grains is required. Errors, 1σ.

as discussed in Supplementary Information. Further time-dependent grain growth and dynamical models will help elucidate the role of light scattered from large-grained dust in the process of mass loss from AGB stars.

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Layered boron nitride as a release layer for mechanical transfer of GaN-based devices

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Nitride semiconductors are the materials of choice for a variety of device applications, notably optoelectronics^{1,2} and high-frequency/high-power electronics³. One important practical goal is to realize such devices on large, flexible and affordable substrates, on which direct growth of nitride semiconductors of sufficient quality is problematic. Several techniques—such as laser lift-off^{4,5}—have been investigated to enable the transfer of nitride devices from one substrate to another, but existing methods still have some important disadvantages. Here we demonstrate that hexagonal boron nitride (h-BN) can form a release layer that enables the mechanical transfer of gallium nitride (GaN)-based device structures onto foreign substrates. The h-BN layer serves two purposes: it acts as a buffer layer for the growth of high-quality GaN-based semiconductors, and provides a shear plane that makes it straightforward to release the resulting devices. We illustrate the potential versatility of this approach by using h-BN-buffered sapphire substrates to grow an AlGaGaN heterostructure with electron mobility of $1,100 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$, an InGaGaN multiple-quantum-well structure, and a multiple-quantum-well light-emitting diode. These device structures, ranging in area from five millimetres square to two centimetres square, are then mechanically released from the sapphire substrates and successfully transferred onto other substrates.

State-of-the-art growth technologies, such as metal–organic vapour phase epitaxy (MOVPE) and molecular beam epitaxy, enable single-crystal high-quality nitride devices to be grown on sapphire, silicon carbide and silicon substrates, but not on polycrystalline or amorphous substrates⁶. Releasing single-crystal high-quality devices from one substrate and transferring them to another is one of the promising ways to address this limitation. In general, nitride semiconductors have been grown on sapphire substrates using a buffer layer: such a layer can consist of low-temperature AlN (ref. 7), low-temperature GaN (ref. 8) or AlON (ref. 9). However, releasing nitride semiconductors from sapphire substrates is difficult owing to the strong covalent sp^3 bonding between the buffer layer and the semiconductors. To overcome this difficulty, thermal release with laser radiation^{4,5}, stamp-based printing¹⁰, chemical release by etching of a sacrificial layer^{11–13}, wet chemical etching^{14–17}, and mechanical release¹⁸ from sapphire substrates have been investigated. Compared with the thermal and chemical approaches, mechanical release is, in principle, an uncomplicated technique requiring neither chemical treatments nor additional equipment for the transfer. However, damage, the limited size of the released GaN, and limited throughput in mechanical releases have remained serious issues. Graphite, graphene¹⁹ and h-BN act as ideal layers for releasing devices from substrates mechanically. However, direct growth of nitride semiconductors on graphene is impossible, and an intermediate layer, such as a zinc oxide (ZnO) nano-wall, is therefore needed¹⁶. In addition, the transfer of nitride semiconductors grown on graphene lacks scalability. On the other hand, we expected that high-quality nitride semiconductors could be grown on h-BN with an AlN or AlGaGaN buffer with high scalability because h-BN is itself a nitride semiconductor and can be grown on a substrate uniformly. In an earlier study, polycrystalline BN films were used as buffer layers to

grow GaN films on (001) Si substrates; however, the grown GaN was polycrystalline²⁰. In addition, the study was restricted to the use of BN as a buffer layer. Here, as a new release layer, we use single-crystal h-BN, which not only acts as a buffer layer for a nitride device but also allows us to release the device from the substrate and transfer it to a foreign substrate mechanically in a highly scalable way. This versatile approach has the potential to be scaled up to production size (several inches) at low cost.

The single-crystal h-BN layer with an atomically flat surface enables us to grow an AlGaGaN heterostructure and InGaGaN-based multiple quantum well (MQW) and MQW LED (light-emitting diode) structures and to release these structures from the host sapphire substrates and transfer them to foreign substrates. Figure 1 depicts our materials design and the release and transfer processes. First, we grew a single-crystal (0001) h-BN ultrathin layer on a (0001) sapphire substrate^{21,22} (Fig. 1a). The orientation relationship between the substrate and the h-BN is $(0001)_{\text{h-BN}} \parallel (0001)_{\text{sapphire}}$ where the plane of boron and nitrogen bonded with sp^2 hybridization is parallel to the substrate surface. Then, the MQW structure—consisting of a 0.3- μm -thick AlGaGaN layer, a 3- μm -thick GaN layer, a ten-period InGaGaN/MQW structure, and a 0.1- μm -thick GaN layer—was grown on the h-BN release layer (Fig. 1a). After the MOVPE growth, we flipped the MQW structure upside down and put it on a foreign substrate via an adhesive sheet (an indium sheet in this case) (Fig. 1b). Next, the MQW structure attached to the indium sheet on the foreign substrate was heated to a temperature sufficient to heat-seal the indium to the sapphire and the MQW. (Fig. 1c). Finally, the MQW structure was released from the host sapphire substrate by mechanical force and the MQW was thereby transferred to the foreign substrate (Fig. 1d). The mechanical force easily separates the MQW from the host sapphire and the separation occurs within the h-BN release layer owing to van der Waals forces of the h-BN layered structure. In this way, MQWs and other types of nitride devices can be transferred to all kinds of substrates, such as silicon, polycrystalline metal, glass and transparent plastics.

An AlN or AlGaGaN layer on the h-BN layer makes it possible to greatly improve the surface morphology and crystalline quality of nitride semiconductors grown on it. GaN directly grown on the h-BN layer has a rough and irregular island-shaped surface morphology, and is polycrystalline (Supplementary Fig. 1). Hence, we first grew a wurtzite AlN layer on the h-BN and then grew a GaN film on the AlN to overcome the difficulty of direct growth of GaN (Fig. 2a). In contrast to GaN grown on the h-BN directly, GaN grown on the AlN shows a step-like flat surface with measured root mean square (r.m.s.) roughness of 0.69 nm over an area of $5 \times 5 \mu\text{m}^2$, as seen in the atomic force microscopy (AFM) image in Fig. 2b. X-ray diffraction in the $2\theta/\omega$ configuration exhibits only GaN (0002) and AlN (0002) diffraction peaks (Fig. 2c), indicating that (0001) single-crystal GaN film has been grown on the (0001) AlN on the h-BN layer. The orientation relationship between the GaN, AlN, h-BN and the sapphire substrate is described in Supplementary Figs 2 and 3. The weak beam dark-field transmission electron microscopy (TEM) images in Fig. 2d and e reveal that the AlN layer, working as a dislocation filter, decreases

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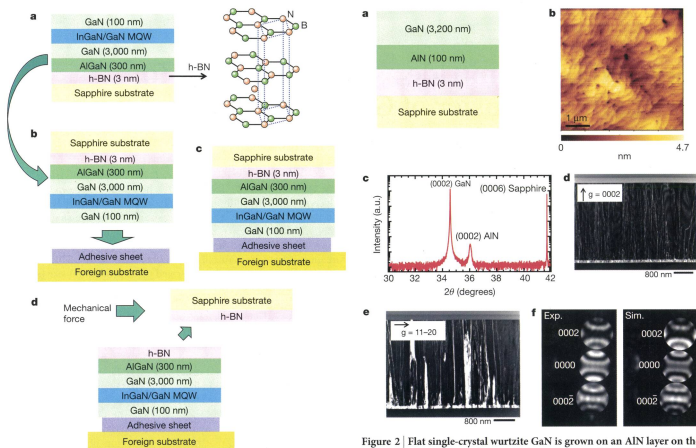


Figure 2 | Flat single-crystal wurtzite GaN is grown on an AlN layer on the h-BN layer. **a**, Schematic illustration. **b**, AFM image of the surface of the GaN film. **c**, X-ray diffraction using the $2\theta/\omega$ configuration for the GaN film. a.u., arbitrary units. **d**, **e**, Weak beam dark-field TEM images. **f**, The reciprocal lattice vector, and the arrows indicate the direction of the vectors. The numbers are Miller indices. **f**, Experimental and simulated CBED patterns. The numbers are Miller indices.

the density of threading dislocations in the GaN and that the predominant defects in the GaN are mixed dislocations with a density of $8.6 \times 10^9 \text{ cm}^{-2}$ (Supplementary Information part III). In the GaN film, the tilt and twist angles were respectively evaluated to be 0.16° and 0.47° from the full-width at half-maximum (FWHM) of the X-ray rocking curve profiles obtained by fitting the rocking curve profiles to a pseudo-Voigt function^{23,24}. Figure 2f shows a convergent beam electron diffraction (CBED) pattern experimentally obtained for the GaN layer and a simulated CBED pattern for Ga-terminated GaN. The good agreement between these patterns reveals that the GaN film has a Ga termination (Fig. 2f). In addition, to verify the device quality of the GaN, we grew an $\text{Al}_{0.28}\text{Ga}_{0.72}\text{N}$ layer with a thickness of 25 nm on the GaN and demonstrated that the AlGaIn/GaN heterostructure with an area of two centimetres square has a two-dimensional electron gas mobility of $1,100 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ with a sheet carrier density of $1 \times 10^{13} \text{ cm}^{-2}$ at room temperature (293 K). Taken together, the AFM, X-ray diffraction, cross-sectional TEM, X-ray rocking curve, CBED and mobility confirm that single-crystal wurtzite GaN of device quality grows on the h-BN layers. In addition, a flat single-crystal GaN layer can be grown on a single-crystal AlGaIn layer on the h-BN (Supplementary Fig. 4). The single crystal h-BN layer works as the release layer. The AlN or AlGaIn is the buffer, and the thick GaN film ensures device quality in spite of the large lattice mismatch.

Before fabricating the LED structure, we grew an InGaIn/GaN MQW structure to investigate its optical properties. The photographs in Fig. 3a and b show the transferred AlGaIn/GaN heterostructure, approximately 2 cm square, and the MQW structure, approximately 5 mm square, on the indium sheets attached to the foreign sapphire substrates, respectively. We can see the surface of the indium sheet because the AlGaIn and the MQW are transparent. The size of the transferred area can be controlled by the adhesive sheet sizes. It takes only a few seconds to release the host sapphire substrate from the AlGaIn/GaN and MQW structure, which is an unusually speedy process compared to laser lift-off. The r.m.s. roughness of the indium sheet is 12 nm over an area of $5 \mu\text{m} \times 5 \mu\text{m}$. Some protrusions from the indium sheet are clearly visible, indicating that the AlGaIn/GaN and MQW structures are mechanically released from the native sapphire substrate, as illustrated in Fig. 1. No cracks were observed in the transparent AlGaIn/GaN structure up to maximum size of about 1 cm square, suggesting that the mechanical release process using the h-BN layer ensures minimal crack formation. The AFM image in Fig. 3c shows that the surface of the transferred structure is flat, with r.m.s. roughness of 0.95 nm over an area of $5 \mu\text{m} \times 5 \mu\text{m}$, which is considerably lower than that of surfaces separated by laser lift-off techniques^{25,26} (typically 10–60 nm). X-ray photoelectron spectroscopy spectra confirm that the separation actually occurs within the h-BN release layer along the plane of B and N bonding (Supplementary Fig. 5). X-ray diffraction (using the $2\theta/\omega$ configuration) measurements of the InGaIn/GaN MQW before and after the transfer showed satellite peaks from the MQW up to first order, along with those for GaN (0002), AlGaIn (0002) and sapphire (Fig. 3d). This indicates that the

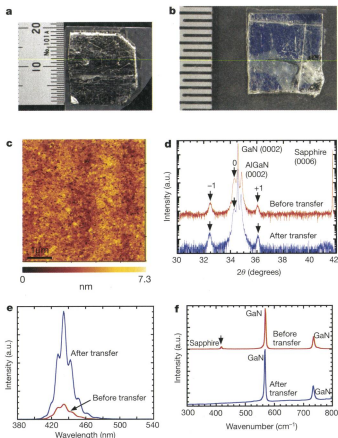


Figure 3 | Structural and optical properties of an InGaN/GaN MQW structure before and after transfer. **a**, Photograph of the transferred AlGaIn structure attached to the indium sheet on the foreign sapphire substrate. The smallest division in the scale at left is 500 μm . **b**, Photograph of the transferred MQW structure attached to the indium sheet on the foreign sapphire substrate. One division in the scale at left is 500 μm . **c**, AFM image of the surface of the transferred structure. **d**, X-ray diffraction using the $2\theta/\omega$ configuration for the InGaN/GaN MQW before and after the transfer. **e**, Photoluminescence spectra at room temperature for the MQW before and after transfer. Excitation wavelength, 375 nm. **f**, Raman spectra for the MQW at room temperature before and after transfer.

MQW period (the sum of well and barrier thickness) is 5.5 nm and that the In content in the InGaN well is 15%. In Fig. 3d, the transferred MQW structure exhibits satellite peaks up to first order with almost the same intensities as those before the release.

To check for degradation of the nitride semiconductor that might be created by release and mounting, we performed room temperature photoluminescence measurements on the MQW before and after the transfer. The excitation source was an InGaN-based semiconductor laser diode with an emission wavelength of 375 nm. Typical photoluminescence spectra from the MQW before and after transfer show strong luminescence at almost the same peak wavelength of 434 nm; however, the intensity after the transfer is comparable to or stronger than that before the transfer (Fig. 3e). We also grew the same InGaN/GaN MQW structure on a GaN film using a standard low-temperature AlN buffer layer on sapphire substrate and performed room-temperature photoluminescence using a He-Cd laser. The luminescence intensity from the as-grown MQW structure on the h-BN release layer was comparable to or higher than that from the one grown on the low-temperature AlN buffer layer. The Raman spectrum for the MQW before the transfer displays the E_2 mode at 569 cm^{-1} and the A_1 mode from the GaN film, and shows the Raman mode from the host sapphire substrate as well (Fig. 3f). The Raman spectrum of the MQW after the transfer shows the E_2 mode from the GaN film at 567 cm^{-1} with a downshift of 2 cm^{-1} (Fig. 3f), indicating that the

GaN before and after the release is compressively strained and unstrained^{27,28}, respectively. The X-ray diffraction, photoluminescence and Raman experiments demonstrate that the MQW structure retains its original crystal quality, even after the transfer processes.

Next, we describe the electroluminescence emitted from the transferred LED (Supplementary Fig. 6a) at room temperature. For comparison, the same MQW LED structure was grown on a typical low-temperature AlN buffer layer on a sapphire substrate and the conventional MQW LED was fabricated without lift-off (Supplementary Fig. 6b). Current-voltage characteristics of the transferred LED show clear rectification (Fig. 4a). In the electroluminescence spectra of the transferred LED and conventional LED with currents ranging from 10 to 50 mA, the intensities of the electroluminescence increase with current (Fig. 4b and c) and the electroluminescence intensities from the transferred MQW LED were comparable to or higher than the intensities from the conventional MQW LED on the low-temperature AlN buffer layer at the same current. These higher electroluminescence intensities are caused by reflection from the back-side contact indium. The comparable intensities and almost the same FWHM of the electroluminescence of the conventional and transferred LED indicate that the MQW preserves its original quality after the transfer. We further succeeded in transferring a vertical-type LED (Supplementary Fig. 6c). This LED emits blue light at room temperature (Fig. 4d).

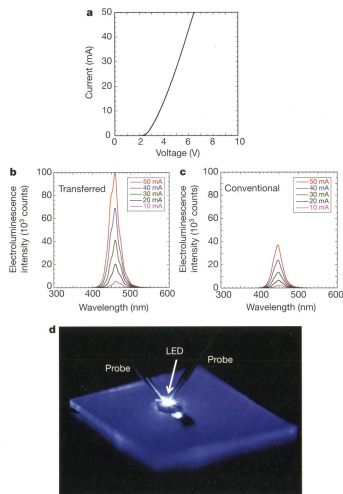


Figure 4 | Electroluminescence of transferred and conventional MQW LED at room temperature. **a**, Current-voltage characteristics of the transferred LED. **b**, Electroluminescence spectra from the transferred LED with currents ranging from 10 to 50 mA. **c**, Electroluminescence spectra from the conventional LED with currents ranging from 10 mA to 50 mA. **d**, Optical image of the blue-light electroluminescence from the transferred vertical-type LED.

Finally, we fabricated a battery-powered LED prototype. The released LED is about 2 mm square and 3.4- μm thick. A schematic cross-section of the LED prototype is shown in Fig. 5a. First, two T-shaped Pd/Au electrodes were deposited on two commercially available laminate films. Then, the released LED structure with the Pd/Au electrodes was mounted on the T-shaped electrode of one laminate film and sandwiched with the other T-shaped electrode of the other laminate film using the indium contact layer. The released LED was thereby sandwiched between the two laminates, which were sealed together by heat. Figure 5b shows a top-view photograph of the LED prototype, which is 18 mm long and 22 mm wide. The side-view of the LED prototype anchored with tweezers shows a thickness of about 200 μm (Fig. 5c), which can offer flexible and portable applications. The LED prototype emitted violet-blue electroluminescence at room temperature (Fig. 5d).

Compared with conventional laser lift-off, mechanical release using h-BN has several advantages. First, this approach requires no lift-off equipment and no chemical etchant, leading to considerable cost reduction. Second, the transfer in this approach is completed within several seconds because the separation harnesses the van der Waals forces of the h-BN release layer. In contrast, laser lift-off requires laser scanning with additional adjustment of the laser. Third, the process is simple: the mechanical force releases GaN-based structures in only one step. In laser lift-off, the laser must be scanned step-by-step, then the irradiated samples must be heated to the melting point of Ga, and finally the GaN sample after laser irradiation must be cleaned with a dilute acid (such as HCl) to remove material residues. The separated surface after the mechanical release is essentially flat, with an r.m.s.

roughness of 1 nm, which is considerably lower than that obtained using laser lift-off. The process we report here opens the way to releasing and transferring a wide range of nitride semiconductor devices to large-area, flexible and affordable substrates.

METHODS SUMMARY

The h-BN release layer, the AlGaIn/GaN heterostructure, the InGaIn/GaN MQW and the MQW LED structures were grown by MOVPE. Triethylboron, trimethylgallium, trimethylaluminum, triethylgallium and trimethylindium were the group III sources and ammonia was the group V source. Silane and bis-cyclopentadienyl-magnesium were the n-type and p-type dopant gases, respectively, with hydrogen or nitrogen carrier gas. Electron beam deposition provided Au, Pd and Al electrodes. A high-resolution X-ray diffractometer (Philips X'Pert System) with a copper target was used to evaluate the structural quality and perform X-ray diffraction pole figure measurement. The photoluminescence measurements were performed with an InGaIn-based laser at a wavelength of 375 nm and a He-Cd laser at room temperature. We used Raman scattering (Renishaw system) with an excitation laser wavelength of 534 nm. We used an electron cyclotron resonance plasma etching system with chlorine to fabricate LEDs.

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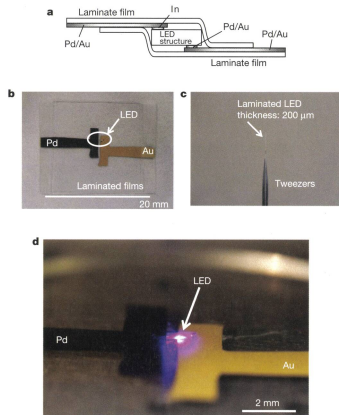


Figure 5 | Electroluminescence of LED prototype at room temperature. a, Schematic cross-section of the LED prototype. b, c, Top-view (b) and side-view (c) photographs of the LED prototype. The T-shaped black Pd electrode surface on one laminate film is on the left-hand side; the gold electrode on the other is on the right-hand side. The LED structure was mounted between the top of the two T-shaped electrodes. d, Optical image of the violet-blue electroluminescence from the LED prototype.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions Y.K. designed the experiments, grew the MQW and LED structures and characterized their structural and optical properties. K.K. fabricated the electrode, released and transferred the LED, and performed the current-voltage and electroluminescence measurements. T.Ä. also grew the MQW and LED structures. T.M. managed the whole experimental process. Y.K. and T.M. proposed this research and wrote the paper.

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Aerosols implicated as a prime driver of twentieth-century North Atlantic climate variability

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Systematic climate shifts have been linked to multidecadal variability in observed sea surface temperatures in the North Atlantic Ocean¹. These links are extensive, influencing a range of climate processes such as hurricane activity² and African Sahel^{3–5} and Amazonian⁶ droughts. The variability is distinct from historical global-mean temperature changes and is commonly attributed to natural ocean oscillations^{6–10}. A number of studies have provided evidence that aerosols can influence long-term changes in sea surface temperatures^{11,12}, but climate models have so far failed to reproduce these interactions^{6,9} and the role of aerosols in decadal variability remains unclear. Here we use a state-of-the-art Earth system climate model to show that aerosol emissions and periods of volcanic activity explain 76 per cent of the simulated multidecadal variance in detrended 1860–2005 North Atlantic sea surface temperatures. After 1950, simulated variability is within observational estimates; our estimates for 1910–1940 capture twice the warming of previous generation models but do not explain the entire observed trend. Other processes, such as ocean circulation, may also have contributed to variability in the early twentieth century. Mechanistically, we find that inclusion of aerosol–cloud microphysical effects, which were included in few previous multimodel ensembles, dominates the magnitude (80 per cent) and the spatial pattern of the total surface aerosol forcing in the North Atlantic. Our findings suggest that anthropogenic aerosol emissions influenced a range of societally important historical climate events such as peaks in hurricane activity and Sahel drought. Decadal-scale model predictions of regional Atlantic climate will probably be improved by incorporating aerosol–cloud microphysical interactions and estimates of future concentrations of aerosols, emissions of which are directly addressable by policy actions.

An understanding of North Atlantic sea surface temperature (NASST) variability is critical to society because historical Atlantic temperature changes are strongly linked to the climate, and its impacts, in neighbouring continental regions. For example, strong links between NASST variability and periods of African Sahel drought are found in observations^{4,13} and physical climate models^{3,5,14}. Similar covariation between NASSTs and rainfall in eastern South America has been found⁵, as have links to changes in both mean rainfall¹⁵ and rainfall extremes¹⁶. Atlantic hurricane activity^{3,10,14} and European summer climate⁶. These changes are not solely limited to the regions bordering the Atlantic, but also have links to Indian monsoon rainfall¹⁴, Arctic and Antarctic temperatures¹⁷, Hadley circulation¹, El Niño/Southern Oscillation¹⁸ and relationships between El Niño/Southern Oscillation and the Asian monsoon¹⁹.

A link between multidecadal variability in NASST and circulation changes internal to the ocean was first proposed in 1964 (ref. 20) and later named the Atlantic Multidecadal Oscillation²¹. This variability is often characterized as the detrended NASST between the equator and latitude 60° N (longitude 7.5–75° W; ref. 8). Although it has recently been questioned²², the present consensus remains that most of the observed Atlantic temperature variations occur in response to the

ocean's internal variability. This picture emerged from general circulation models, a number of which inherently produce multidecadal Atlantic variability in the absence of external climate forcing² and, when considered together as a multimodel mean, have shown little evidence of forced changes projecting onto the NASSTs^{6,9}. Observationally, this interpretation has been accepted because the Atlantic temperature changes seem to be oscillatory, both around any secular long-term trend and when calculated as anomalies from the global-mean change.

Motivated by the recent identification of the importance of aerosol process complexity in interhemispheric Atlantic temperature changes²³, apparent aerosol correlation^{11,12} and volcanic modulation of Atlantic variability²⁴, we use new general circulation model simulations to question whether the CMIP3 (Climate Model Intercomparison Project phase 3) models contained the complexity necessary to represent a forced Atlantic Multidecadal Oscillation^{7,9}. We use HadGEM2-ES (the Hadley Centre Global Environmental Model version 2 Earth System configuration²⁵), a next-generation CMIP5 (Climate Model Intercomparison Project phase 5) model, which represents a wider range of Earth system processes (in particular aerosol interactions²⁵) than do CMIP3 models.

To separate internal variability from forced changes, we present climate model ensemble-mean NASSTs, averaged over parallel model simulations started from different initial conditions²⁶. If external forcing dominates the NASST evolution then ensemble members will evolve in phase and thus combine to produce a robust ensemble-mean response. If internal ocean dynamics dominate then each member will evolve separately and the resulting ensemble mean will show little residual variation around the underlying warming trend. This approach allows identification of physical mechanisms linking forced changes to Atlantic temperatures and was used in previous CMIP3 studies^{6,9}.

In Fig. 1a, we reproduce the multimodel-mean NASST response of the six CMIP3 models used in ref. 9 (ENS1, blue) and the eleven models used in ref. 6 (ENS2, green) (Supplementary Table 2). The observations (Fig. 1) show marked multidecadal variations. The multimodel-mean responses in both ENS1 and ENS2 do capture the underlying trend through the century; they capture only weak multidecadal variability. For example, the ensembles' 1950–1975 cooling is only a small fraction of the observed value (Fig. 1a and Supplementary Fig. 4). Therefore, the unexplained multidecadal signal was previously attributed to internal ocean variability^{6,9}.

By contrast, HadGEM2-ES (Fig. 1b) reproduces much more of the observed NASST variability (correlation, 0.65; 75% of detrended standard deviation (smoothed over 10-yr intervals to highlight multidecadal component)). The post-1950s cooling and subsequent warming now falls within the observed trends (Supplementary Table 1). Observed warming in the earlier period (1910–1940) is larger than simulated by HadGEM2-ES (Fig. 1b and Supplementary Table 1); however, these new simulations capture roughly twice the early-twentieth-century warming of previous CMIP3 generation models.

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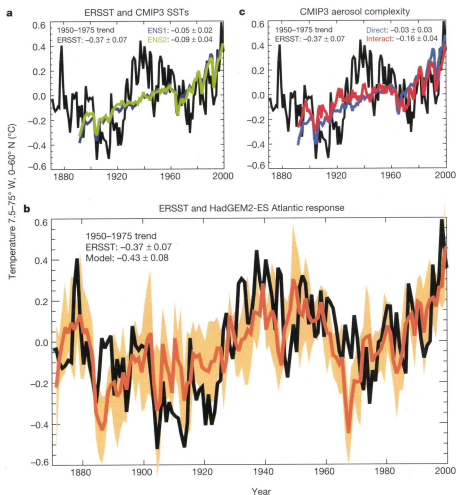


Figure 1 | Atlantic surface temperatures. Comparison of the area-averaged North Atlantic SSTs (defined as 7.5–75° W and 0–60° N), relative to the 1901–1999 average, of an observational estimate (the US National Oceanic and Atmospheric Administration's Extended Reconstructed SST¹⁷ (ERSST), black) and two published¹⁸ CMIP3 model composites (ENS1, blue; ENS2, green; a); the HadGEM2-ES model (orange; shading represents 1 s.d. of the model ensemble spread; b); and two recomposites from CMIP3, the first with models

that represent only direct aerosol (mean of five contributing models, red) and the second with models representing both indirect effects interactively (three models, blue). (c). In all panels, trends between 1950–1975 (K per decade) are shown. The error estimates are based on the s.d. of the 25 trends between a 5-yr period (1948–1952) at the start of this interval and a 5-yr period (1973–1977) at the end. All data have been latitude-weighted when calculating area averages.

This points to a larger forced role in this period. Other processes not represented by our ensemble-mean response (such as ocean dynamical changes) may also contribute to this early trend.

In examining why the HadGEM2-ES ensemble reproduces the observed NASST variability better than previous multimodel studies have done^{6,9} (Fig. 1a, b), we can discount the possibility that the HadGEM2-ES variability is predetermined, because the initial conditions were selected to sample different phases of Atlantic variability²⁶. Furthermore, an additional HadGEM2-ES ensemble that omits changes in aerosol emissions neither has the same multidecadal variability as the all-forcings ensemble nor reproduces the observed NASSTs (Fig. 2a).

Replication of a large fraction of the observed NASST variability by HadGEM2-ES allows us to identify forcings and mechanisms, consistent with the observed variability, within the model framework. Variability of ensemble-mean NASST from historical simulations including time-varying aerosol emissions is strongly correlated with variability in simulated net surface shortwave radiation (Fig. 2b), which in turn has the same temporal structure as variability in aerosol optical depth changes (Fig. 2c) and periods of volcanic activity (Fig. 2d). Other terms in the surface heat budget (Supplementary Fig. 2) have a role in the simulated NASST change. However, it is the surface shortwave component that produces the dominant multidecadal variations.

Volcanoes and aerosols respectively explain 23 and 66% of the temporal (10-yr-smoothed) multidecadal variability of the detrended NASST (Supplementary Fig. 5). Combining both contributions explains 76% (80% after inclusion of mineral dust aerosols) of the simulated variance. Inclusion of mineral dust processes may potentially be important because emissions are known to respond to North-Atlantic-driven changes in Sahel rainfall, and thus represent an important positive feedback on NASSTs in the real world¹². The lack of a multidecadal dust signal (Supplementary Information) in HadGEM2-ES simulations suggests that we are likely to be underestimating the magnitude of the forced Atlantic response.

The volcanic influence on Atlantic variability has been demonstrated previously^{12,22}. We focus on the anthropogenic aerosol component of the shortwave changes identified here as driving the model's multidecadal NASST variability. Aerosol concentration changes influence the spatial response (Fig. 3) of NASST as well as its temporal evolution. Prevailing winds advect aerosols emitted in industrial North America in a band across the North Atlantic that mixes with polluted air masses over Europe before being transported by trade winds south and west. The large-scale pattern of shortwave change is explained by the effect of cloud microphysical response to these changes in aerosol concentration. The shortwave variability largely occurs where aerosol changes coincide with large-scale cloud distribution. On a regional

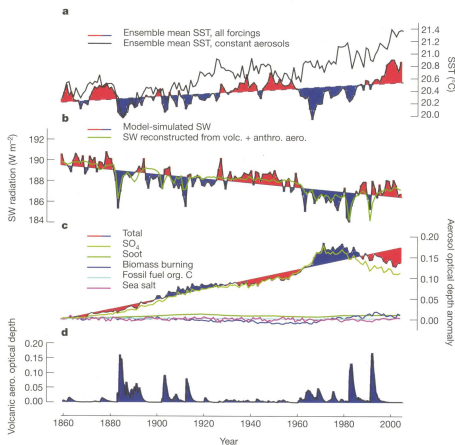


Figure 2 | External forcing of surface temperature and surface shortwave radiation linked to aerosol and volcanic changes. **a**, Ensemble-mean NASST (7.5–75° W and 0–60° N) simulated by the HadGEM2-ES model considering all available external climate forcings (red/blue), and all forcings except anthropogenic aerosol emissions (black). **b**, Ensemble-mean shortwave (SW) radiation entering ocean (red/blue) alongside reconstruction of this shortwave radiation based on a linear relationship with total anthropogenic aerosol and

volcanic optical depth changes (green). The linear model explains 79% of the simulated variance. **c**, Change in total (red/blue shading) and individual species (coloured lines) of anthropogenic aerosol optical depth (degree of absorption/scattering) over the North Atlantic. **d**, Volcanic optical depth from ref. 30 as implemented in HadGEM2-ES simulations. In **a–c**, red and blue shading represents values above or below (or vice versa) a least-squares linear fit to the data.

scale, coupled processes, such as temperature feedbacks, can lead to an enhanced local response (Supplementary Information). The same horseshoe-shaped signature is seen in shortwave and NASST variability (Fig. 3). The map of NASST change between warm and cold phases of multidecadal variability is consistent with the observed variations in SSTs (Fig. 3).

So far consistent spatial and temporal changes between aerosol burden, shortwave and NASST have been presented. It is not clear, however, whether these changes are externally forced by aerosols or are mediated by ocean circulation. Here we present a parallel simulation of the historical period, driven by identical emission and concentration changes, but with the SSTs explicitly fixed at their 1860 climatological values. The shortwave changes arising in this parallel experiment share the temporal structure and magnitude of the shortwave changes from our standard historical simulations (Fig. 4a). By explicitly removing any feedback from SST change on shortwave, we demonstrate that simulated historical shortwave variability arises directly from aerosol and volcanic forcing of the surface radiation and is not mediated by ocean circulation change.

One of the reasons why the role of aerosols in driving multidecadal variability has not previously been identified is the level of aerosol physics represented in climate models at the time of the CMIP3 multi-model comparison project (Supplementary Table 2). Although all the CMIP3 models represented the direct effect of aerosols on shortwave radiation, most omitted or only partly represented the indirect aerosol effects²³.

Recently, albedo differences in CMIP3 aerosol representation have been shown to be important for simulating SST changes²³: models that represent indirect aerosol effects capture more of the observed Atlantic interhemispheric change than those that do not. Recombining the models used in Fig. 1a into those with only direct aerosol effects, and those that also include the first indirect effect interactively, shows some evidence of multidecadal variability (Fig. 1c), illustrating that aerosol–cloud microphysical processes have a role even in previous-generation models. These models do not, however, reproduce the magnitude of the multidecadal NASST of HadGEM2-ES.

In HadGEM2-ES, the aerosol indirect effects account for 80% or more of the total aerosol forcing in the North Atlantic region (Fig. 4b and Supplementary Fig. 1). Although there is some discussion of the magnitude of the indirect effects^{28,29}, omission of these processes will lead to an underestimation of the modelled aerosol impact on the NASST. Looking at the relative roles of the first and second indirect effects (using changes in optical depth and cloud effective radius as respective metrics for these effects; Fig. 4c), we see a more pronounced response to early-twentieth-century variations for the indirect effect (effective radius) due to higher sensitivity of cloud albedo changes to changes in aerosol number in cleaner conditions⁴. In all, the inclusion of aerosol indirect effects in HadGEM2-ES magnifies the shortwave and, hence, the NASST response to aerosols, as well as influencing the spatial and temporal character of the historical multidecadal variability. We also note that although some climate models (such as HadGEM2-ES) reproduce the observed sensitivity of cloud albedo to changes in

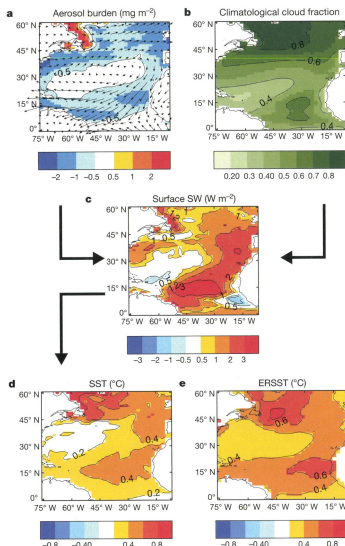


Figure 3 | Differences in spatial response between warm and cold Atlantic phases. Differences between the average of warm years (warmest third of the data) and the average of cold years (coldest third of the data), after the data has been detrended, show how patterns of multidecadal variability in aerosol burden (a) interact with the climatological cloud field (b) to influence the pattern of net surface shortwave radiation change (c), and hence, NASSTs (d). The pattern of aerosol burden changes is linked to emissions in industrial North America and Europe by the climatological wind field (the direction and magnitude of which is indicated by the arrows). The exception is the localized increase in aerosol burden (Canadian coast) driven by increases in sea salt aerosols (warm years reduce sea ice extent in this area). The warm phase/cold phase SST pattern simulated by the model (d) agrees well with the observed change (e). Vertical axes show latitude; horizontal axes show longitude.

aerosol optical depth in maritime regions, not all parameterizations of aerosol indirect effects do so (Fig. 2e in ref. 29).

We have shown that volcanic and aerosol processes can drive pronounced multidecadal variability in historical NASST, which leads to improved (for the early twentieth century) or reproduces (for the later period) the observed historical trends. In these simulations, it is the inclusion of aerosol indirect effects that allows us to capture the magnitude and the temporal and spatial structure of SST variability. Our results show that volcanoes and, crucially (from a policy and climate impact perspective), anthropogenic emissions of aerosols can drive NASST variability resembling that which is observed. This work suggests that we need to reassess the current attribution to natural ocean variability of a number of prominent past climate impacts linked to NASSTs, such as Sahel drought.

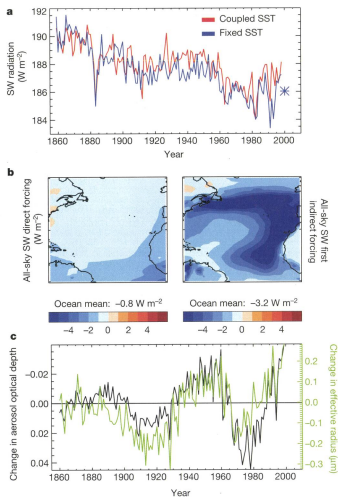


Figure 4 | Magnitude and origin of forced changes in net surface shortwave radiation. a, Ensemble-mean time evolution of surface shortwave radiation in the North Atlantic region (red; see also Fig. 2b) and the surface shortwave from a parallel simulation in which the SSTs were held fixed at climatological values from 1860 (blue). The comparison shows that these shortwave changes are externally forced. These long-term trends in surface shortwave match the changes observed in a snapshot experiment (2000 aerosol emissions, 1860 SSTs; blue asterisk), indicating that the underlying trend is consistent with that expected from aerosol changes alone. b, Spatial patterns of surface shortwave forcing arising from the 2000 direct effect (left) and the first indirect effect (right) of aerosols (Supplementary Information), which illustrate the dominant role of indirect effects in the total forcing and the spatial distribution. c, Detrended time series of aerosol optical depth (black) and cloud-top effective radius (green) from the coupled simulations, which are indicators of the temporal evolution of direct and, respectively, indirect effects. Although the variations in effective radius are largely in phase with those in optical depth, there is greater divergence (implying a larger role for indirect effects) in the early historical period.

METHODS SUMMARY

The climate model used in this study is HadGEM2-ES²⁴. This model is notable for the number of climate-biogeochemical interactions that are interactively calculated rather than specified in advance. Of relevance to this work, HadGEM2-ES models the supply of oxidants, an important component for aerosol formation, and mineral dust aerosols interactively, and yields improved predictions of biomass and carboniferous aerosol properties. Source terms for natural aerosols (or precursors) and mineral dust are also modelled interactively. Each simulation in the ensemble is forced with driving data (greenhouse gases, aerosols, volcanoes, land use and solar changes) based on historical data sets compiled for CMIP5 simulations. These data sets and their implementation within this model are extensively documented in ref. 26. Volcanic forcing is prescribed in latitudinal bands. Over the North Atlantic, the magnitude of optical depth changes are prescribed individually for the bands spanning 0–30° N and 30–90° N, capturing the differences between tropical and extratropical volcanoes. Individual members of

the ensemble were initiated from a control simulation using start points located 50 yr apart¹⁰.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Adaptation at the output of the chemotaxis signalling pathway

Junhua Yuan¹, Richard W. Branch¹, Basarab G. Hossu¹ & Howard C. Berg¹

In the bacterial chemotaxis network, receptor clusters process input^{1–3}, and flagellar motors generate output⁴. Receptor and motor complexes are coupled by the diffusible protein CheY-P. Receptor output (the steady-state concentration of CheY-P) varies from cell to cell⁵. However, the motor is ultrasensitive, with a narrow operating range of CheY-P concentrations⁶. How the match between receptor output and motor input might be optimized is unclear. Here we show that the motor can shift its operating range by changing its composition. The number of FlIM subunits in the C-ring increases in response to a decrement in the concentration of CheY-P, increasing motor sensitivity. This shift in sensitivity explains the slow partial adaptation observed in mutants that lack the receptor methyltransferase and methylesterase^{7,8} and why motors show signal-dependent FlIM turnover⁹. Adaptive remodelling is likely to be a common feature in the operation of many molecular machines.

The chemotaxis signalling pathway allows bacterial cells to sense and respond to changes in concentrations of chemical attractants or repellents^{1,2}. Binding of chemicals by receptors modulates the activity of an associated histidine kinase, CheA, thereby changing the level of phosphorylation of the response regulator, CheY. CheY-P binds to FlIM, a component of the switch complex at the base of the flagellar motor and modulates the direction of motor rotation. A phosphatase, CheZ, dephosphorylates CheY-P. The chemotaxis pathway is well known for its high gain^{8,10,11}, wide dynamic range^{11,12} and robust adaptation^{5,13}, mediated by receptor methylation and demethylation (by CheR and CheB).

The output of the chemotaxis pathway, the flagellar motor, is ultrasensitive to the intracellular concentration of CheY-P, with a Hill coefficient of about 10, imposing a narrow operational range for [CheY-P]⁶. Whereas precise adaptation is a robust property of the chemotaxis pathway for certain attractants, for example aspartate, the steady-state concentration of CheY-P is not⁵. Given cell-to-cell variations in the concentration of CheY-P and the fact that different cells can maintain their chemotactic sensitivity¹⁴, it has been suggested that cells might have additional molecular mechanisms to adjust the CheY-P concentration around the operational value of approximately 3 μ M⁶. One possibility is a feedback mechanism that allows a cell to adjust its kinase activity in response to motor output. This mechanism would increase the kinase activity if cells only ran, and would decrease the kinase activity if cells only tumbled. In earlier work, we looked for such a mechanism by monitoring the kinase activity with a fluorescence resonance energy transfer (FRET) technique¹⁵ while jamming flagellar bundles with an anti-filament antibody. Stopping motors had no effect on kinase activity¹⁶.

Here we report that the motor itself adapts, shifting its response function according to the steady-state concentration of CheY-P. It does this by increasing the complement of FlIM when the concentration of CheY-P is low. Motor remodelling is well known for the stator elements MotA and MotB, which if defective, can be replaced by wild-type protein, as evidenced by stepwise increments in motor torque^{17,18}. Such exchange also has been visualized by total internal reflection

fluorescence (TIRF) microscopy of green fluorescent protein (GFP)-labelled protein¹⁹, and a similar technique has been used to demonstrate FlIM⁹ and FlIN²⁰ exchange in cells containing CheY-P. The present work addresses the functional consequences of FlIM exchange. We studied *cheR cheB* cells, which are defective in methylation and demethylation, and monitored motor and kinase responses to step-addition of the non-metabolizable attractant α -methylaspartate (MeAsp), using bead²¹ and FRET¹⁵ assays. These experiments cannot be done with wild-type cells because their adaptation to aspartate is robust, so that the steady-state concentration of CheY-P does not change. Motor adaptation occurs on a minute rather than on a second timescale and does not play a direct role in sensing temporal gradients. Instead, it helps to match the operating point of the motor to the output of the chemotaxis receptor complex, obviating the requirement for fine-tuning of that output.

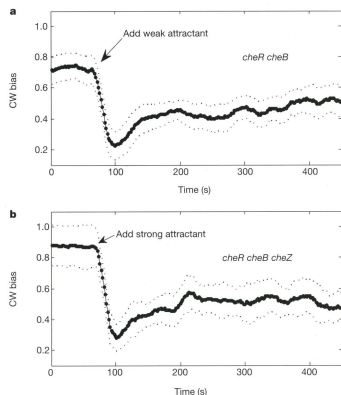


Figure 1 | Motor responses to stepwise addition of chemical attractants monitored by the bead assay. The attractants were applied at the times indicated by the arrows. Error bounds for standard errors of the mean are shown as dotted lines. **a**, Averaged responses of seven *cheR cheB* cells (JY35 carrying pKAF131) to 1 mM MeAsp (weak attractant). **b**, Averaged responses of four *cheR cheB cheZ* cells (JY32 carrying pVS7 and pKAF131) to 2 mM MeAsp + 0.5 mM L-serine (strong attractant).

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Using a bead assay, we found partial adaptation in *cheR cheB* cells within 1 min following the initial response, Fig. 1a, which shows the averaged responses of seven motors on different cells to stepwise addition of 1 mM MeAsp. These results are similar to those obtained previously with tethered cells^{8,9}. A recent model suggests that partial adaptation might be due to dynamic localization of CheZ²². To test this hypothesis, we repeated the bead experiments using *cheR cheB cheZ* cells. The results were essentially the same; Fig. 1b shows the averaged responses of four motors on different cells of a *cheR cheB cheZ* strain to stepwise addition of 2 mM MeAsp + 0.5 mM L-serine (a stronger stimulus needed because of the lower sensitivity of *cheZ* strains). So CheZ is not required for this partial adaptation.

CheY-P concentrations were monitored by measuring FRET between cyan fluorescent protein (CFP)-conjugated CheZ (CheZ-CFP) and yellow fluorescent protein (YFP)-conjugated CheY (CheY-YFP). We measured responses in *cheR cheB* cells to stepwise addition of 1 mM MeAsp, Fig. 2a. The response shown in Fig. 2a is similar to that obtained previously¹¹. No adaptation is apparent. To rule out possible complications due to CheZ oligomerization²³, we also measured CFP-Fluorimetry/CheY-YFP FRET²⁴ in *cheR cheB* cells following stepwise addition of 2 mM MeAsp + 0.5 mM L-serine (a stronger stimulus needed because of the lower sensitivity of CFP-Fluorimetry/CheY-YFP FRET), as shown in Fig. 2b. No adaptation is apparent in either panel of Fig. 2, so the partial adaptation shown in Fig. 1 does not occur upstream of CheY-P. It must occur at the level of the flagellar motor.

Clockwise (CW) biases of motors were measured before addition of attractant, immediately after addition of attractant, and after time was allowed for partial adaptation. We focused on motors with pre-stimulus CW biases around 0.8 (ranging from 0.70 to 0.95). Owing to cell-to-cell variation, the lowest biases following stimulation ranged from 0 to 0.75. The concentration of CheY-P in a given cell was estimated from its CW bias at the time of the lowest bias, using the response curve measured previously⁶, shown by the red line in Fig. 3 (Hill coefficient $N_H = 10.3$, CheY-P dissociation constant $K = 3.1 \mu\text{M}$). Then the CW bias found after that cell had adapted was plotted as a

function of this concentration, as shown by the blue data points in Fig. 3. The measurements were carried out for 49 motors on different *cheR cheB* cells with stepwise addition of 0.5 or 1 mM MeAsp. Following adaptation, the relationship between the CW bias and the concentration of CheY-P shifted to lower concentrations of CheY-P, increasing motor sensitivity to CheY-P.

How the motor accomplishes this shift is intriguing. We sought to explain the shift in the motor response curve by using a Monod-Wyman-Changeux (MWC) type model^{25,26}, which has been used previously to explain the motor switching kinetics²⁶. In this model, the C-ring is considered to be an allosteric switch, stochastically switching between two conformational states, counterclockwise (CCW) and CW, with N independent binding sites for CheY-P, corresponding to N units of FlhM in the C-ring. The CW state has a higher affinity to CheY-P than the CCW state. The CW bias of the motor is given by $B_{CW} = (1 + [\text{CheY-P}]/K)^N / (1 + [\text{CheY-P}]/K)^N + L(1 + [\text{CheY-P}]/(KC))^N$, where L is the ratio of the probability that the motor is in the CCW state to the probability that it is in the CW state in the absence of CheY-P, K is the CheY-P dissociation constant for the CW state, and C is the ratio of dissociation constants for the CCW and CW states, respectively²⁶. With reasonable values for the parameters, for example, $N = 34$, $L = 10^3$ and $K = 3.1 \mu\text{M}$ ^{6,27}, the model can be fit to the ultrasensitivity data of ref. 6 with a best-fit value of C of 4.1, as shown by the red curve in Fig. 3. With these values for the parameters L , K and C , and assuming that the number of FlhM units N varies with $[\text{CheY-P}]$, we can fit the data measured for the adapted motor using the MWC model with $N = N_{av} + \alpha([\text{CheY-P}] - 2.7)$, where N is written as a Taylor expansion about the average value of $[\text{CheY-P}]$, $2.7 \mu\text{M}$. We obtain a two-parameter fit with $N_{av} = 36$ and $\alpha = -1.2 \mu\text{M}^{-1}$, shown by the green curve in Fig. 3. The average value of N has increased from 34 to 36. Sensitivity of an MWC complex is known to increase with N for fixed values of L , K and C (ref. 28). Equivalently, the motor bias versus $[\text{CheY-P}]$ curve shifts to smaller $[\text{CheY-P}]$ with larger N as shown in Fig. 3. Intriguingly, the fact that increasing the number of FlhM units causes an increase in CW bias can be understood by considering the energetics of the switch. Each CheY-P binding decreases the energy level of CW state by a specific amount. With the values for parameters L , K and C fixed, increasing the number of FlhM, that is, CheY-P binding sites, increases the number of CheY-P bound to the motor.

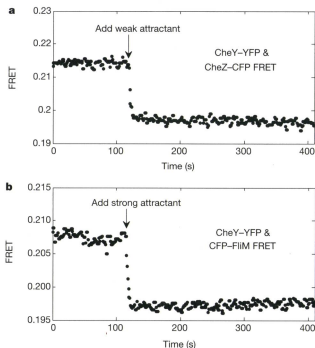


Figure 2 | FRET responses (Y/C ratio) of *cheR cheB* cells to stepwise addition of chemical attractants. The attractants were added at the times indicated by the arrows. a, CheY-YFP/CheZ-CFP FRET responses to 1 mM MeAsp (weak attractant). b, CheY-YFP/CFP-Fluorimetry FRET responses to 2 mM MeAsp + 0.5 mM L-serine (strong attractant).

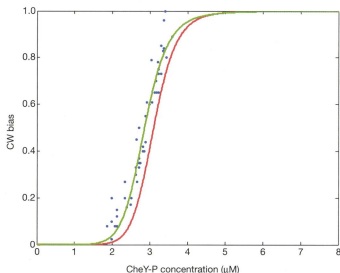


Figure 3 | CW bias as a function of CheY-P concentration. The red curve is for pre-stimulus wild-type motors, as measured in ref. 6 and fit with an MWC model with 34 FlhM units²⁶. The blue dots are data for motors that have partially adapted to stepwise addition of attractant, with a two-parameter fit to the MWC model with $N_{av} = 36$ FlhM units shown by the green curve; see text.

This decreases the energy level of the CW state, thereby increasing the CW bias.

To test directly for this increase of the number of FliM units, we fused YFP to the carboxy terminus of FliM and monitored the fluorescence intensity of single motors using TIRF microscopy. To minimize shifts in motor position, we tethered a *cheR cheB* strain that lacks the flagellar filament to glass via single hooks with anti-hook antibody. Changes in fluorescence were measured upon addition of 2 mM MeAsp + 0.5 mM L-serine, which should saturate the chemoreceptors and eliminate CheY-P, or of 1 mM MeAsp, which should simply reduce the concentration of CheY-P. The results are shown in Fig. 4. Figure 4a is the averaged response of 20 motors to the strong attractant, and Fig. 4b is the averaged response of 22 motors to the weak attractant. In either case, the fluorescence intensity increased following the addition of attractant on a time scale consistent with partial adaptation, by a larger amount for the stronger attractant. We compensated for fluorescence bleaching by subtracting a control curve (Fig. 4c) and fitting the results to a model in which the FliM off rate decreases when

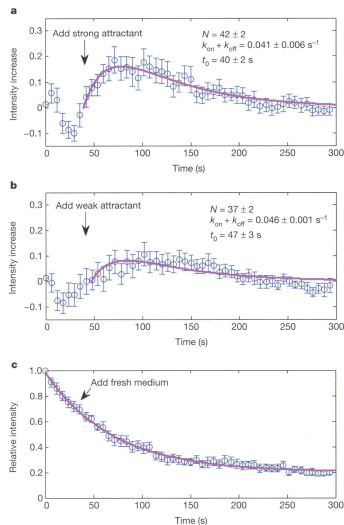


Figure 4 | Changes in single-motor FliM-YFP fluorescence intensities in *cheR cheB* cells tethered by hooks and stimulated by addition of attractant (at time t_0 , arrow). a–c, The intensity for a given motor was normalized by its intensity at time 0, intensities for different motors were averaged, and the fit to the control of panel c was subtracted. Fits with the model are shown in magenta and the parameters for these fits are given. Error bars are standard errors of the mean. a, Responses of 20 motors to addition of 2 mM MeAsp + 0.5 mM L-serine. b, Responses of 22 motors to addition of 1 mM MeAsp. c, Responses of 15 motors without addition of attractant, fit with an exponential decay function plus a constant.

the CheY-P concentration decreases; see Methods. Steady state is reached when $Nk_{off} = (M - N)k_{on}$, where N is the number of FliM molecules in the motor, and M is the maximum number of FliM binding sites in the motor. The fits are shown in magenta and the final values for N are given in each panel (assuming an initial value of 34). These values agree with those presented in Fig. 3. So the motor increases the number of FliM units as it partially adapts to a decrement in the concentration of CheY-P. By doing so, it increases its dynamic range.

We eliminated the concern that binding of CheY-P to FliM-YFP might be different than binding to wild-type FliM by using the bead assay to compare the biases, switching rates and speeds for motors of *cheR cheB* cells expressing FliM-YFP or wild-type FliM: the biases were $61 \pm 16\%$ or $58 \pm 19\%$, the switching rates were 3.8 ± 1.2 or $3.4 \pm 1.0 \text{ s}^{-1}$, and the speeds were 50.4 ± 8.4 or $51.5 \pm 8.2 \text{ Hz}$, respectively.

The motor adaptation mechanism observed here is related to the turnover of motor C-ring components discovered recently²⁰, where exchange of FliM was found to be signal-dependent and exhibited a similar timescale²¹. The detailed mechanism should involve changes in FliM on/off rates dependent upon either CheY-P binding or rates of motor switching. As noted earlier, the timescale for motor adaptation (1 min) is much slower than that for receptor methylation/demethylation (1 s), which enables cells to make rapid temporal comparisons; thus, motor adaptation does not play a critical role in that aspect. Instead, it helps match the operating point of the motor to the output of the chemotaxis receptor complex.

METHODS SUMMARY

All strains used in this study were derivatives of *Escherichia coli* K12 strain RP437. Cells were grown at 33°C in 10 ml T-broth supplemented with the appropriate antibiotics and inducers to an $A_{600 \text{ nm}}$ of 0.45 to 0.50. Cells were collected by centrifugation (10 min at 1,300g), washed twice in 10 ml of motility medium (10 mM potassium phosphate/0.1 mM EDTA/1 μM methionine/10 mM lactic acid, pH 7.0), and resuspended in 10 ml of this medium. They were used immediately for experiments or stored at 4°C for up to 2 h. All experiments were carried out with a custom-made flow chamber at room temperature.

For the bead assay, cells were sheared to truncate flagella, and 1.0- μm -diameter polystyrene latex beads were attached to the filament stubs. Rotation of the bead was monitored with a laser dark-field setup described previously²². Rotational velocity as a function of time was determined for each motor and smoothed with a 25-point running average. CW bias was calculated over a 20-s interval every 2 s, leading to a plot of CW bias versus time.

FRET measurements of bacterial populations were carried out as described previously²³.

For TIRF measurements, cells were tethered to the bottom window of the flow chamber by single hooks using anti-FliG antibody, following a protocol adapted from ref. 21. The fluorescence intensity of the motors was monitored with a TIRF microscope (Nikon Eclipse Ti-U), and images were recorded with a back-illuminated, cooled (-55°C), electron-multiplying CCD camera (DV887ECS-BV, Andor Technology). Image analysis of the motor spots was carried out using a Gaussian mask method described previously¹⁸.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Trans-synaptic Teneurin signalling in neuromuscular synapse organization and target choice

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Synapse assembly requires trans-synaptic signals between the pre- and postsynapse¹, but our understanding of the essential organizational molecules involved in this process remains incomplete². Teneurin proteins are conserved, epidermal growth factor (EGF)-repeat-containing transmembrane proteins with large extracellular domains³. Here we show that two *Drosophila* Teneurins, Ten-m and Ten-a, are required for neuromuscular synapse organization and target selection. Ten-a is presynaptic whereas Ten-m is mostly postsynaptic; neuronal Ten-a and muscle Ten-m form a complex *in vivo*. Pre- or postsynaptic Teneurin perturbations cause severe synapse loss and impair many facets of organization trans-synaptically and cell autonomously. These include defects in active zone apposition, release sites, membrane and vesicle organization, and synaptic transmission. Moreover, the presynaptic microtubule and postsynaptic spectrin cytoskeletons are severely disrupted, suggesting a mechanism whereby Teneurins organize the cytoskeleton, which in turn affects other aspects of synapse development. Supporting this, Ten-m physically interacts with α -Spectrin. Genetic analyses of *teneurin* and *neuroligin* reveal that they have differential roles that synergize to promote synapse assembly. Finally, at elevated endogenous levels, Ten-m regulates target selection between specific motor neurons and muscles. Our study identifies the Teneurins as a key bi-directional trans-synaptic signal involved in general synapse organization, and demonstrates that proteins such as these can also regulate target selection.

Vertebrate teneurins are enriched in the developing brain^{4,5}, localize to synapses *in culture*⁶, and pattern visual connections⁷. Both *Drosophila* Teneurins, Ten-m and Ten-a, function in olfactory synaptic partner matching⁸ and were further identified in neuromuscular junction (NMJ) defect screens^{9,10}, with Ten-m also affecting motor axon guidance¹¹. We examine their roles and the underlying mechanisms involved in synapse development.

Both Ten-m and Ten-a were enriched at the larval NMJ (Fig. 1a and Supplementary Fig. 1a). Ten-a was detected at neuronal membranes; this staining was undetectable beyond background in *ten-a* null mutants (Supplementary Fig. 1b) and barely detectable after neuronal Ten-a RNA interference (RNAi; Supplementary Fig. 1c), indicating that Ten-a is predominantly presynaptic. Partial co-localization was observed between Ten-a and the periaxial zone marker Fasciclin 2 (ref. 12) as well as the active zone marker Bruchpilot¹³ (Fig. 1b, c), suggesting that Ten-a is localized to the junction between the periaxial zone and the active zone. Ten-m appeared strongly postsynaptic and surrounded each bouton (Fig. 1a and Supplementary Fig. 1a, d). Muscle-specific *ten-m* RNAi eliminated the postsynaptic staining, but uncovered weak presynaptic staining (Supplementary Fig. 1e) that ubiquitous *ten-m* RNAi eliminated (Supplementary Fig. 1f). Thus, the Ten-m signal was specific and, although partly presynaptic, enriched postsynaptically. Consistently, muscle Ten-m colocalized extensively with Dlg (Fig. 1d) and completely with α -Spectrin (Fig. 1e) and is thus probably coincident with all postsynaptic membranes.

The localization of Ten-a and Ten-m suggested their trans-synaptic interaction. To examine this, we co-expressed Myc-tagged Ten-a in nerves using the Q system¹⁴ and haemagglutinin (HA)-tagged Ten-m in muscles using GAL4. Muscle Ten-m was able to co-immunoprecipitate nerve Ten-a from larval synaptosomes (Fig. 1f), suggesting that the Teneurins form a heterophilic trans-synaptic receptor pair at the NMJ.

To determine Teneurin function at the NMJ, we examined the *ten-a* null allele and larvae with neuron or muscle RNAi of *ten-a* and/or *ten-m*. Following such perturbations, bouton number and size were altered: the quantity was reduced by 55% (Fig. 2a–c, g and Supplementary Fig. 2)

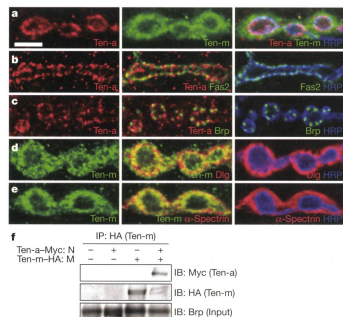


Figure 1 | Teneurins are enriched at and interact across *Drosophila* neuromuscular synapses. a–e, Representative single confocal sections of synaptic boutons stained with antibodies against Ten-a (red) or Ten-m (green), horseradish peroxidase (HRP) to mark the neuronal membrane (blue), and a synaptic marker as indicated. a, Ten-a is associated with presynaptic membranes and Ten-m largely with the surrounding postsynapse (a). b, c, Ten-a shows limited co-localization with the periaxial zone marker Fasciclin 2 (b), and Bruchpilot (Brp), an active zone marker (c). d, e, Ten-m co-localizes with, and extends beyond, Dlg (d) and completely co-localizes with muscle α -Spectrin (e). f, Immunoblots (IB) of larval synaptosomes expressing neuronal Flag-Myc-tagged Ten-a (N) and muscle Flag-HA-tagged Ten-m (M) and immunoprecipitated (IP) using antibodies to HA. Ten-a is detected in the pull-down, indicating that nerve Ten-a and muscle Ten-m interact across the NMJ. This is not seen in control lanes. Owing to low expression, neither transgene product is detectable in input lysates, which are enriched in Brp. Scale bar, 5 μ m.

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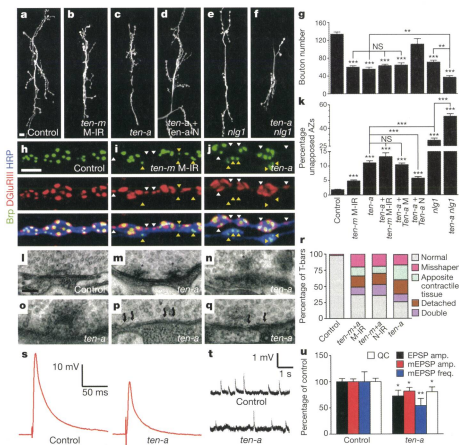


Figure 2 | Teneurins affect the structure and function of the neuromuscular synapse. **a–f**, Representative NMJs stained with antibodies to HRP. Muscle-specific *ten-m* RNAi (M-IR) and lots of *ten-a* decrease bouton number. Neuronal *ten-a* RNAi (N-R) but not muscle (*Ten-a* M) restoration of *ten-a* expression rescues this phenotype. These defects resemble *nlg1* mutants and are enhanced in *ten-a nlg1* double mutants. **g**, Quantification of bouton number. **h–j**, Representative NMJs stained with antibodies to Brp (green), the glutamate receptor subunit DGluRIII (red) and HRP (blue). In control larvae (**h**), Brp and DGluRIII puncta properly appose. *teneurin* perturbations (**i**, **j**) disrupt this active zone (yellow arrowhead) and glutamate receptor apposition (white arrowhead). **k**, Quantification of unopposed active zone/glutamate receptor pairs. For each quantification, $n \geq 8$ larvae, 16 NMJs. **l–q**, Transmission electron microscopy of active zone T-bars (asterisks) in control larvae (**l**) and

ten-a mutants (**m–q**) showing double T-bars (**m**), detached T-bars (**n**), misshapen T-bars (**o**), membrane ruffling (**p**, wavyed arrows) and T-bars facing contractile tissue (**q**). Some images show multiple defects. **r**, Distribution of T-bar defects as a percentage of the total T-bars. N-R, neuronal RNAi. For each genotype, $n \geq 3$ larvae, 40 boutons. **s**, **t**, Representative evoked EPSP (**s**) and mEPSP (**t**) traces from control and *ten-a* mutant genotypes. **u**, Quantification of mean EPSP amplitude (black), mEPSP amplitude (red), mEPSP frequency (blue) and quantal content (QC, white) expressed as a percentage of the control average. For all genotypes, $n \geq 7$ larvae, 8 muscles. Error bars represent s.e.m. Scale bars, 10 μ m (**a–f**), 5 μ m (**h–j**), 100 nm (**l–q**). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, NS, not significant. Statistical comparisons are with control unless noted.

and the incidence of large boutons markedly increased (Supplementary Fig. 2k). Both changes indicate impaired synaptic morphogenesis. The reduction in bouton number was probably cumulative through development, as it was visible in first instar *ten-a* mutants and persisted (Supplementary Fig. 2k). In the *ten-a* mutant, bouton morphogenesis was rescued by restoring *Ten-a* expression in neurons, but not muscles (Fig. 2d, g and Supplementary Fig. 2). Neuronal *Ten-m* overexpression could not substitute for the lack of *Ten-a*, revealing their non-equivalence (Supplementary Fig. 2e, i). Neuronal knockdown of *Ten-a* or *Ten-m* resulted in fewer synaptic boutons (Supplementary Fig. 2f–h, i), indicating that both have a presynaptic function, although presynaptic *Ten-a* has a more predominant role (Supplementary Fig. 2l). Moreover, knocking down postsynaptic *Ten-m* in the *ten-a* mutant did not enhance the phenotype (Fig. 2g). Thus, presynaptic *Ten-a* (and, to a lesser extent, *Ten-m*) and postsynaptic *Ten-m* are required for synapse development.

teneurin perturbation also caused defects in the apposition between presynaptic active zones (release sites) and postsynaptic glutamate receptor clusters¹⁸ (Fig. 2h and Supplementary Fig. 3): up to 15% of the active zones/receptor clusters lacked their partner compared to 1.8% in controls (Fig. 2h–k). Under electron microscopy, active zones

are marked by electron-dense membranes and single presynaptic specializations called T-bars (Fig. 2l), which enable synapse assembly, vesicle release and Ca^{2+} -channel clustering¹⁹. Teneurin disruption caused defects (Fig. 2m–r and Supplementary Fig. 3) in T-bar ultrastructure (Fig. 2m–o), membrane organization, and apposition to contractile tissue (Fig. 2p, q). Teneurin perturbation also impaired postsynaptic densities while increasing membrane ruffling (Supplementary Table 1), further indicating organizational deficiency. These phenotypes resemble mutants with adhesion and T-bar biogenesis defects^{17,18}, suggesting a role for Teneurins in synaptic adhesion and stability. Synaptic vesicle populations similarly required Teneurins for clustering at the bouton perimeter and proper density (Supplementary Fig. 4). As these effects are not synonymous with active zone disruption¹⁹, Teneurins are also required for synaptic vesicle organization.

Synapses lacking *teneurin* were also functionally impaired. The mean amplitude of evoked excitatory postsynaptic potentials (EPSPs) in larvae was decreased by 28% in the *ten-a* mutant (Fig. 2s, u). Spontaneous miniature EPSPs showed a 20% decrease in amplitude, a 46% decrease in frequency (Fig. 2t, u), and an altered amplitude distribution compared with control (Supplementary Fig. 5a). These defects resulted in a 20% reduction in quantal content (Fig. 2u), which could be partly due to

fewer boutons and release sites. However, release probability may also be reduced, as suggested by an increased paired pulse ratio in *ten-a* mutants (Supplementary Fig. 5d, e). The decay kinetics of responses were faster in *ten-a* mutants, suggesting additional postsynaptic effects on glutamate receptors and/or intrinsic membrane properties (Supplementary Fig. 5b, c). Further, FM1-43 dye loading revealed markedly defective vesicle cycling in *ten-a* mutants (Supplementary Fig. 5f, h). Consistent with physiological impairment, *teneurin*-perturbed larvae exhibited profound locomotor defects (Supplementary Fig. 5i). In summary, Teneurins are required for multiple aspects of NMJ organization and function.

As a potential mechanism for synaptic disorganization following *teneurin* perturbation, we examined the pre- and postsynaptic cytoskeletons. In the presynaptic terminal, organized microtubules contain Futsch (a microtubule-binding protein)-positive 'loops', whereas disorganized microtubules possess punctate, 'unbundled' Futsch²⁰. Each classification normally represented ~10% (often distal) of boutons (Fig. 3a, d and Supplementary Fig. 6). Upon *teneurin* perturbation, many more boutons had unbundled Futsch (Fig. 3b, c and Supplementary Fig. 6) whereas those with looped microtubules were decreased by 62–95% (Fig. 3d). Therefore, proper microtubule organization requires pre- and postsynaptic Teneurins. In contrast to mild active zone/glutamate receptor apposition defects, most boutons displayed microtubule organizational defects.

teneurin perturbation also severely disrupted the postsynaptic spectrin cytoskeleton, with which Ten-m colocalized (Fig. 1e). Postsynaptic α -Spectrin normally surrounds the bouton (Fig. 3e). Perturbing neuronal or muscle Teneurins markedly reduced postsynaptic α -Spectrin without affecting Dlg (Fig. 3f–h and Supplementary Fig. 7). Postsynaptic β -Spectrin²¹, Adducin²² and Wsp were similarly affected (Supplementary Fig. 8). In muscle, α -Spectrin is coincident with and essential for the integrity of the membranous subsynaptic reticulum (SSR)^{21,23}. Consistent with this, *teneurin* disruption reduced SSR width up to 70% (Supplementary Fig. 9d–g) and increased the frequency of 'ghost' boutons, which are failures of postsynaptic membrane organization²³

(Supplementary Fig. 9a–d). Thus, Teneurins are involved in the organization of the pre- and postsynaptic cytoskeletons and postsynaptic membranes. Further, endogenous α -Spectrin co-immunoprecipitated with muscle-expressed, Flag-tagged Ten-m (Fig. 3i), suggesting that Ten-m physically links the synaptic membrane to the cytoskeleton.

Because the most severe defects following *teneurin* perturbation were cytoskeletal, we propose that Teneurins primarily organize the presynaptic microtubule and postsynaptic spectrin-based cytoskeletons (Fig. 3j), which then organize additional synaptic aspects^{20,21}. However, such a solitary role cannot fully explain the observed phenotypes. The reduction in bouton number associated with cytoskeletal disruption is milder than that following *teneurin* disruption^{20,21,24}. Also, although active zone dynamics are affected by cytoskeletal perturbation²¹, defects in apposition are not^{21,25}. Moreover, the T-bar structural defects more closely resemble synapse adhesion and active zone formation defects^{17,18}. Thus, Teneurins may regulate release site organization and synaptic adhesion independent of the cytoskeleton (Fig. 3j).

Our data also indicate that Teneurins act bi-directionally across the synaptic cleft. Ten-a acts predominantly in neurons, as evidenced by localization, phenotypes caused by neuronal (but not muscle) knock-down, and mutant rescue by neuronal (but not muscle) expression (Figs 2 and 3 and Supplementary Figs 2–4, 6, 7 and 9). Yet, in addition to the presynaptic phenotypes, many others were postsynaptic, including reduced muscle spectrin, SSR, and membrane apposition (Fig. 3 and Supplementary Figs 7–9). Similarly, although Ten-m is present both pre- and postsynaptically, muscle knockdown resulted in presynaptic defects, including microtubule and vesicle disorganization, reduced active zone apposition, and T-bar defects (Figs 2 and 3 and Supplementary Figs 3, 4, 6 and 7). Thus, Teneurins function in bi-directional trans-synaptic signalling to organize neuromuscular synapses. This may involve downstream pathways or simply establish an organizational framework by the receptors themselves. Moreover, as the results of single disruptions of neuronal *ten-a* or muscle *ten-m* were similarly severe and not enhanced by combination (Figs 2g and 3d, h and Supplementary Fig. 2k), both Ten-a and Ten-m probably

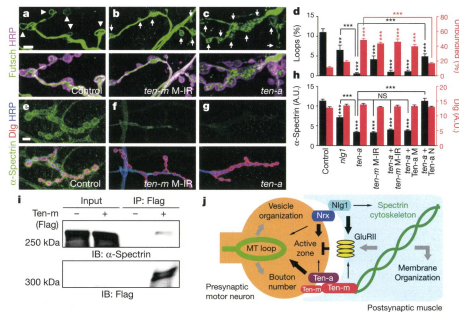


Figure 3 | Teneurin perturbation results in marked cytoskeletal disorganization. a–c, Representative NMJs stained with antibodies to Futsch (green) and HRP (magenta). Arrowheads indicate looped organization. Arrows indicate unbundled Futsch. d, Quantification of the percentage of total boutons with looped or unbundled microtubules. e–g, Representative NMJs stained with antibodies to α -Spectrin (green), Dlg (red) and HRP (blue). Following *teneurin* perturbation, α -Spectrin staining is largely lost. Axonal α -Spectrin is unaffected by muscle *teneurin* RNAi (f). h, Quantification of α -Spectrin (green)

and Dlg (red) fluorescence. A.U., arbitrary units. For all genotypes, $n \geq 6$ larvae, 12 NMJs. i, Immunoblots (IB) showing that α -Spectrin is detected in the Flag immunoprecipitates (IP) of larvae expressing muscle Flag-HA-tagged Ten-m but not in control larvae. Owing to low expression, Flag-HA-Ten-m is only detectable after enrichment by immunoprecipitation. j, Model showing the roles of Teneurins, Neuroxin and Neuroligin at the NMJ. Arrow size represents the relative contribution of each pathway to the cellular process as inferred from mutant phenotypic severity. Scale bars, 5 μ m. *** $P < 0.001$, NS, not significant.

function in the same pathway. Our finding that Ten-a and Ten-m co-immunoprecipitate from different cells *in vitro*⁸ and across the NMJ *in vivo* (Fig. 1f) further suggests a signal via a trans-synaptic complex. Teneurin function, however, may not be solely trans-synaptic. In some cases (vesicle density, SSR width), cell-autonomous knockdown resulted in stronger phenotypes than knocking down in synaptic partners (Supplementary Figs 3, 4, 9 and Supplementary Table 1). This suggests additional cell-autonomous roles in addition to trans-synaptic Teneurin signalling.

Signalling involving the transmembrane proteins Neurexin and Neuroligin also mediates synapse development²⁹. In *Drosophila*, Neurexin (*nrx*) and Neuroligin 1 (*nlg1*) mutations cause phenotypes similar to *teneurin* perturbation: reductions in bouton number, active zone organization, transmission, and SSR width^{27,28}. *nlg1* and *nrx* mutations do not enhance each other, suggesting that they function in the same pathway²⁹. Consistently^{27,28}, we found that *nrx* and *nlg1* mutants exhibited largely similar phenotypes (data not shown). To investigate the relationship between the *teneurin* genes and *nrx* and *nlg1*, we focused on the *nlg1* null mutant. Both Nlg1 tagged with enhanced green fluorescent protein (Nlg1-eGFP) and endogenous Ten-m occupied a similar postsynaptic space (Supplementary Fig. 10a). *teneurin* and *nlg1* loss-of-function mutations also displayed similar bouton number reductions (Fig. 2e, g), vesicle disorganization (Supplementary Fig. 4), and ghost bouton frequencies (Supplementary Fig. 9). Other phenotypes showed notable differences in severity. In *nlg1* mutants, there was a 29% failure of active zone/glutamate receptor apposition (Fig. 2k and Supplementary Fig. 10d), compared to 15% for the strongest *teneurin* perturbation. The cytoskeleton of *nlg1* mutants, however, was only mildly impaired compared to that seen with *teneurin* perturbations (Fig. 3d, h and Supplementary Figs 6 and 7).

To examine further the interplay of *teneurin* and *nlg1*, we analysed *ten-a nlg1* double mutants. Both single mutants were viable, despite their synaptic defects. Double mutants, however, were larval lethal. We obtained rare escapers, which showed a 72% reduction in boutons, compared to a 50–55% decrease in single mutants (Fig. 2e). Active zone apposition in double mutants was enhanced synergistically over either single mutant (Fig. 2k and Supplementary Fig. 10e). Cytoskeletal defects in the double mutant resembled the *ten-a* mutant (Fig. 3 and Supplementary Figs 6 and 7). These data suggest that *teneurin* genes and *nrx* and *nlg1* act in partially overlapping pathways, cooperating to organize synapses properly, with Teneurins contributing more to cytoskeletal organization and Neurexin and Neuroligin to active zone apposition (Fig. 3j).

In the accompanying manuscript⁸, we showed that although the basal Teneurins are broadly expressed in the *Drosophila* antennal lobe, elevated expression in select glomeruli mediates olfactory neuron partner matching. At the NMJ, this basal level mediates synapse organization. Analogous to the antennal lobe, we found elevated *ten-m* expression at muscles 3 and 8 using the *ten-m-GAL4* enhancer trap (Fig. 4a). We confirmed this for endogenous *ten-m*, and determined that it was contributed by elevated Ten-m expression in both nerves and muscles (Fig. 4b–g). Indeed, *ten-m-GAL4* was highly expressed in select motor neurons, including MN3-Ib, which innervates muscle 3 (ref. 29; Supplementary Fig. 11c). This elevated larval expression also varied along the anterior–posterior axis (Supplementary Fig. 12), and was specific for Ten-m, as Ten-a expression did not differ within or between segments (data not shown).

To test whether elevated Ten-m expression in muscle 3 and MN3-Ib affects neuromuscular connectivity, we expressed *ten-m* RNAi using *ten-m-GAL4*. Wild-type muscle 3 was almost always innervated (Fig. 4h). However, after *ten-m* knockdown, muscle 3 innervation failed in 11% of hemisegments (Fig. 4i, j). This required Ten-m on both sides of the synapse, as the targeting phenotype persisted following neuronal or muscle RNAi suppression using tissue-specific *GAL80* transgenes (Fig. 4j). *ten-a* RNAi did not show this phenotype (Fig. 4j), consistent with homophilic target selection via Ten-m. The phenotype

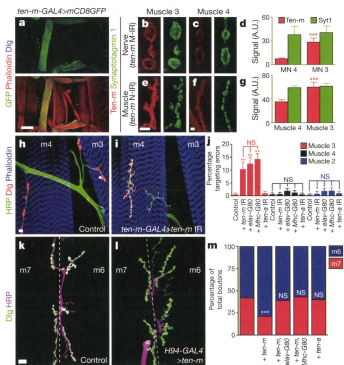


Figure 4 | High-level Ten-m expression regulates muscle target selection.

a, Representative images of hemisegment A3 stained with antibodies to Dlg (blue), phalloidin (red), and expressing GFP via *ten-m-GAL4* (green). High-level expression is observed in muscles 3 and 8 and basally in all muscles. **b, c**, Muscle 3 (**b**) and 4 (**c**) NMJs show differential Ten-m (red) but similar Syntrophin 1 (Syt1; green) expression (from a *ten-m* muscle knockdown animal). **d**, Quantification of presynaptic Ten-m (red) and Syt1 (green) fluorescence at muscle 3 and 4 NMJs. MN, motor neuron. **e, f**, NMJs at muscles 3 (**e**) and 4 (**f**) show differential Ten-m (red) but similar Syt1 (green) expression in muscles (from a *ten-m* nerve knockdown). **g**, Quantification of postsynaptic Ten-m (red) and Syt1 (green) fluorescence at muscle 3 and 4 NMJs. **h, i**, Representative images stained with phalloidin (blue) and antibodies to HRP (green) and Dlg (red) to visualize motor neurons and muscles in control (**h**) or *ten-m-GAL4>ten-m* larvae (**i**). **j**, Quantification of the hemisegment percentage with failed muscle 3 (**red**), 4 (**black**) or 2 (**blue**) innervation. IR, interfering RNA. **k, l**, Representative images of the muscle 6/7 NMJ labelled with antibodies to Dlg (green) and HRP (magenta). The characteristic wild-type arrangement of boutons (**k**) is shifted towards muscle 6 when Ten-m is overexpressed in that muscle and the innervating motor neurons (**l**). **m**, Quantification of the total bouton percentage on muscles 6 (blue) and 7 (red). All genotypes contain *H94-GAL4*, additional transgenes are indicated (for details, see Methods). The Ten-m-mediated shift is abolished by neuronal or muscle *GAL80* transgenes. Scale bars, 100 μ m (**a**), 5 μ m (**b–i**), 10 μ m (**k, l**). In all cases, $n \geq 12$ larvae. **** $p < 0.0001$, *** $p < 0.001$, NS, not significant.

was specific to muscle 3, as innervation onto the immediately proximal or distal muscle was unchanged (Fig. 4j). The low penetrance is probably due to redundant target selection mechanisms³⁰. Where innervation did occur, the terminal displayed similarly severe phenotypes to other NMJs (not shown). Thus, in addition to generally mediating synaptic organization, Ten-m also contributes to correct target selection at a specific NMJ.

To determine whether Ten-m overexpression could alter connectivity, we expressed Ten-m in muscle 6 (but not the adjacent muscle 7), and the motor neurons innervating both muscles using *H94-GAL4*. Normally, 60% of the boutons at muscles 6/7 are present on muscle 6 with 40% on muscle 7 (Fig. 4k, m). Ten-m overexpression caused a shift whereby 81% of boutons synapsed onto muscle 6 and only 19% onto muscle 7 (Fig. 4l, m). This shift also required both neuronal and muscle Ten-m, as neuronal or muscle *GAL80* abrogated it (Fig. 4m). The effect was specific because Ten-a overexpression did not alter this synaptic balance (Fig. 4m), nor was it secondary to altered bouton

number, which was unchanged (data not shown). Therefore, elevated Ten-m on both sides of the NMJ can bias target choice. This, combined with evidence that Ten-m can mediate homophilic interaction *in vitro*⁸, supports a trans-synaptic homophilic attraction model at the NMJ as in the olfactory system.

We identified a two-tier mechanism for Teneurin function in synapse development at the *Drosophila* NMJ. At the basal level, Teneurins are expressed at all synapses and engage in hetero- and homophilic bi-directional trans-synaptic signalling to organize synapses properly (Fig. 3j). Supporting this, Teneurins can mediate homo- and heterophilic interactions *in vitro*⁸ and heterophilic interactions *in vivo* (Fig. 1f). At the synapse, Teneurins organize the cytoskeleton, interact with α -Spectrin, and enable proper adhesion and release site formation. Further, elevated Ten-m expression regulates target selection in specific motor neurons and muscles via homophilic matching and functions with additional molecules³⁰ to mediate precise neuromuscular connectivity. Teneurin-mediated target selection at the NMJ is analogous to its role in olfactory synaptic partner matching⁸. As Teneurins are expressed broadly throughout the antennal lobe, it remains an attractive possibility that they also regulate synapse organization in the central nervous system.

METHODS SUMMARY

Details of *Drosophila* stocks, immunostaining, electron microscopy, functional assays, construction of epitope-tagged Teneurin constructs, immunoprecipitation, imaging and statistical analysis can be found in Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions T.J.M. designed and performed all experiments (apart from electrophysiology), W.H. characterized and provided new reagents, and assisted in some experiments. V.S.D. and T.J.M. designed and V.S.D. performed electrophysiology experiments with assistance from T.J.M. V.F. provided new reagents. L.L. supervised the project. T.J.M. wrote the manuscript with feedback from all authors.

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Transcription factor PIF4 controls the thermosensory activation of flowering

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Plant growth and development are strongly affected by small differences in temperature¹. Current climate change has already altered global plant phenology and distribution^{2,3}, and projected increases in temperature pose a significant challenge to agriculture⁴. Despite the important role of temperature on plant development, the underlying pathways are unknown. It has previously been shown that thermal acceleration of flowering is dependent on the florigen, FLOWERING LOCUS T (FT)^{5,6}. How this occurs is, however, not understood, because the major pathway known to upregulate FT, the photoperiod pathway, is not required for thermal acceleration of flowering⁶. Here we demonstrate a direct mechanism by which increasing temperature causes the bHLH transcription factor PHYTOCHROME INTERACTING FACTOR4 (PIF4) to activate FT. Our findings provide a new understanding of how plants control their timing of reproduction in response to temperature. Flowering time is an important trait in crops as well as affecting the life cycles of pollinator species. A molecular understanding of how temperature affects flowering will be important for mitigating the effects of climate change.

Arabidopsis thaliana, like many higher plants, responds to warmer ambient temperatures by increasing its growth rate and accelerating the floral transition^{1,5,7}. *Arabidopsis* is a facultative long-day plant, and plants grown under short photoperiods are dramatically delayed in flowering. Interestingly, late flowering in short days can be overcome by growth at higher temperatures⁸. The underlying mechanism is, however, unknown. The flowering response to temperature is dependent on the floral pathway integrator gene *FT*⁹, indicative of a thermosensory pathway that upregulates FT expression independently of daylength. Because the bHLH transcription factor PIF4 has been shown to regulate architectural responses to high temperature^{8,9}, we tested if PIF4 is required for the induction of flowering at high temperature in short photoperiods. Although *pif4-101* was slightly delayed in flowering at 22 °C, *pif4-101* mutants showed a striking loss of thermal induction of flowering at 27 °C (Fig. 1a, b). To test if *pif4-101* perturbed floral induction by affecting FT expression, we examined the thermal induction of FT in Col-0 and *pif4-101*. Although FT expression was strongly thermally inducible in Col-0, this response was largely abolished in *pif4-101* at 27 °C (Fig. 1c), indicating that PIF4 is necessary for the thermal acceleration of flowering in short days. By contrast, PIF4 is not required for the thermosensory induction of flowering under continuous light⁸, suggesting that the photoperiod pathway also interacts with the ambient temperature sensing pathway. The reduced role of PIF4 under continuous light probably reflects the instability of PIF4 in light¹⁰ coupled with the fact that the output of the photoperiod pathway, CONSTANS (CO) protein, is stabilized by light¹¹, shifting the balance of floral induction from PIF4 to the photoperiod pathway. Because PIF4 is necessary for the thermal induction of flowering in short days, we tested if it is sufficient to trigger flowering when overexpressed. 35S::PIF4 caused extremely early flowering

(Fig. 1d, e), similar to the effect of overexpressing a related gene, PHYTOCHROME INTERACTING FACTOR5 (ref. 12), suggesting that PIF4 is limiting for the acceleration of flowering at lower temperature in short photoperiods. Consistently, 35S::PIF4 plants showed elevated levels of FT (Fig. 1f). Furthermore, 35S::PIF4 *ft-3* showed a complete suppression of the early flowering phenotype, indicating that the induction of flowering by 35S::PIF4 was dependent on FT (Fig. 1g, h). This activation of FT appears to be independent of the established

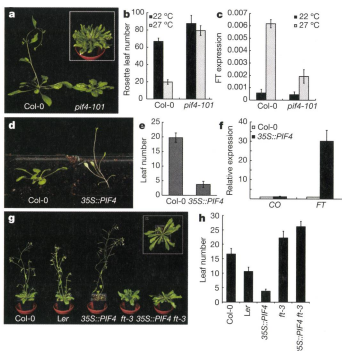


Figure 1 | PIF4 is necessary for the thermal induction of flowering in short photoperiods. **a**, *pif4-101* plants do not show acceleration of flowering at 27 °C compared with Col-0. Inset, a 16-week-old *pif4-101* plant grown at 27 °C. **b**, Rosette leaf numbers at flowering for Col-0 and *pif4-101* grown at 22 and 27 °C in short-photoperiod conditions (error bars, SD; *n* = 6). **c**, FT expression as measured by quantitative polymerase chain reaction (qPCR) PCR in 4-week-old plants at 22 and 27 °C under short photoperiods in a PIF4-dependent manner (data from three biological replicates; error bars, SD). **d**, 35S::PIF4 overexpression triggers very early flowering. **e**, Leaf numbers at flowering for Col-0 and 35S::PIF4 in long photoperiods (error bars, SD; *n* = 5). **f**, CO and FT gene expression data measured by qPCR in Col-0 and 35S::PIF4 at 21 °C in long photoperiods (samples taken 2 weeks after sowing; data from three biological replicates; all error bars, SD). **g**, FT is required for the early flowering phenotype of 35S::PIF4. When crossed into the *ft-3* background, the early flowering of 35S::PIF4 is completely suppressed. Inset, top view of the 35S::PIF4 *ft-3* plant showing that petiole elongation growth is retained. **h**, Flowering time data for Col-0, Ler, 35S::PIF4, *ft-3* and 35S::PIF4 *ft-3* plants (error bars, SD; *n* = 5).

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photoperiod pathway because *CO* did not change in response to 35S::PIF4 (Fig. 1f). Finally, although *co-9* mutants are late flowering^{6,13}, we found 35S::PIF4 *co-9* plants were early flowering, indicating that *PIF4* acts largely independently of *CO* (Supplementary Information and Supplementary Fig. 1), consistent with the thermal induction of flowering being independent of the photoperiod pathway (Fig. 1f).

Although *PIF4* has been shown to be important for high-temperature responses, long-term increases in either *PIF4* transcript or *PIF4* protein levels in response to higher ambient temperature that can account for the observed growth responses have not been detected^{8,9}. To examine if variation of *PIF4* transcription under our experimental conditions might account for the increases in *PIF4* activity with temperature, we measured *PIF4* transcript levels at 12, 17, 22 and 27 °C in seedlings (Fig. 2a). *PIF4* transcript levels increased from 12 °C to 22 °C, whereas the difference between 22 °C and 27 °C was not statistically significant. Plants at 27 °C, compared with 22 °C, showed a very large *PIF4*-dependent response, suggesting that variation in the *PIF4* transcript is not sufficient to account for the acceleration of flowering at 27 °C compared with 22 °C. To test whether temperature-mediated changes in *PIF4* transcription are rate limiting for the biological response, we analysed the behaviour of plants constitutively expressing *PIF4*. Although 35S::PIF4 plants at 22 °C were extremely early flowering, this phenotype could be largely suppressed at 12 °C (Fig. 2b and Supplementary Fig. 2), indicating that even when *PIF4* transcript is abundant, lower temperatures are inhibitory for *PIF4* activity. A possible explanation for this difference is that *PIF4* protein is destabilized by low temperature. Indeed, *PIF4* protein levels have already been shown to be strongly regulated by light¹⁰, and growth in red and blue photocycles destabilizes *PIF4* protein at low temperatures¹¹. The *PIF4* over-expression lines contain a fusion to the haemagglutinin (HA) epitope (35S::PIF4:HA). We therefore examined the levels of PIF4:HA protein at 12, 17, 22 and 27 °C under the same light conditions used for our flowering time assays. Consistent with previous studies¹⁰ we saw a strong accumulation of *PIF4* at the end of the night period, which was subsequently degraded during the day. Despite the suppression of early flowering in 35S::PIF4 at 12 °C compared with 22 °C (Fig. 2b), we did not observe an appreciable difference in *PIF4* protein levels at

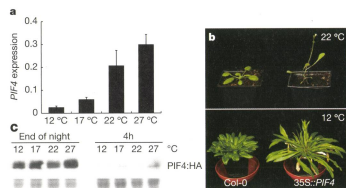


Figure 2 | Regulation of *PIF4* by temperature. **a**, Transcriptional regulation of *PIF4* by temperature. Ten-day-old Col-0 seedlings grown at 12, 17, 22 and 27 °C under short photoperiods were analysed for *PIF4* expression by qPCR. Data shown are from three biological replicates. Error bars, s.d. **b**, Flowering phenotype of 35S::PIF4 is temperature dependent. Although 35S::PIF4 plants (right) flower very early at 22 °C (upper panel) compared with Col-0 (left), this phenotype is largely suppressed by growth at 12 °C (lower panel). **c**, *PIF4* protein levels in 35S::PIF4 plants are not affected by growth temperature. Seven-day-old 35S::PIF4 seedlings grown at 17 °C were transferred to 12, 17, 22 and 27 °C under short photoperiods for 2 days and samples were collected at the end of night before light and after 4 h under illumination. Although *PIF4*:HA protein levels are independent of growth temperatures, the protein is robustly degraded in presence of light. *PIF4*:HA protein was detected by horseradish peroxidase (HRP)-conjugated HA antibody. Stained lower half of the gel used for immunoblot is shown as loading control.

these two temperatures that was likely to account for these different phenotypes (Fig. 2c and Supplementary Fig. 3). Slightly higher levels of *PIF4*:HA appeared to be present at 27 °C (Fig. 2c), suggesting high-temperature stabilization of *PIF4* may also contribute to higher *PIF4* activity at 27 °C.

Taken together, these data indicate that *PIF4* regulates *FT* in a temperature-dependent manner. To determine if this is probably the case in *planta*, we analysed the spatial expression of *FT* and *PIF4*. *FT* has a distinctive pattern of expression in the vasculature of the leaf^{15,16}, and significantly *PIF4* was expressed in the same domain (Fig. 3a). Because the regulation of *FT* by *PIF4* could be either direct or indirect, we used chromatin immunoprecipitation (ChIP) to analyse if *PIF4* binds directly to the *FT* promoter proximal to the transcriptional start site. This region of the promoter was chosen because it has been shown to be both phylogenetically conserved and the site for light-mediated regulation of *FT* expression^{16,17}. We observed robust enrichment of *PIF4* near to the transcriptional start site (Fig. 3b), indicating that *PIF4* binds this region *in vivo* to activate *FT* expression.

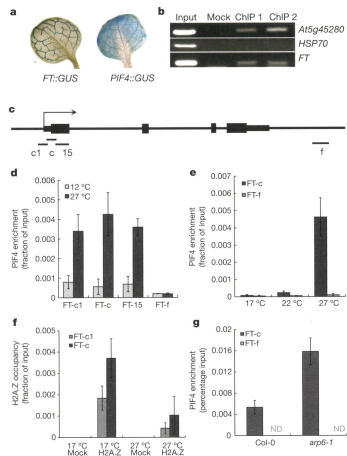


Figure 3 | *PIF4* directly binds the *FT* promoter in a temperature-dependent manner. **a**, β -Glucuronidase (GUS) histochemical analysis of the expression domains of *FT* and *PIF4* in rosette leaves. **b**, ChIP analysis shows *PIF4* binding to the *FT* locus *in vivo* in seedlings. The *At5g45280* promoter is a positive control for *PIF4*-binding activity¹¹, the *HSP70* promoter was used as a negative control. **c**, Summary of the structure of the *FT* promoter and positioning of qPCR amplicons for ChIP analysis. **d**, ChIP analysis of 35S::PIF4:HA at 12 and 27 °C (2-week-old seedlings, short photoperiods). **e**, ChIP analysis of *PIF4*:PIF4:ProA at 17, 22 and 27 °C (4-week-old soil-grown plants, short photoperiods). **f**, Analysis of H2A.Z occupancy at the *FT* locus at 17 and 27 °C (3-week-old plate-grown plants, short photoperiods). **g**, ChIP analysis of *PIF4* binding to *FT* in Col-0 and *arap6-1* (3-week-old soil-grown plants, 22 °C short photoperiod). (For all ChIP experiments, plant materials were collected at the end of dark period before lights came on and were protected from light until frozen. All data presented are from two independent ChIP experiments; all error bars, SD.)

Given the striking effect of ambient temperature on PIF4 activity, which occurs even when *PIF4* is constitutively expressed, we hypothesized that the ability of PIF4 to bind the *FT* promoter may be temperature dependent. To test this, we performed ChIP experiments using 35S::*PIF4* plants grown at 12 and 27 °C with primers flanking an E-box in the *FT* promoter (Fig. 3c). Strikingly, we observed a very strong temperature dependence for this binding, with an approximately fivefold increase in binding at 27 °C compared with 12 °C (Fig. 3d). This indicates that the later flowering of 35S::*PIF4* at 12 °C is caused by a decrease in PIF4 binding to *FT*. Because the 35S promoter causes strong ectopic expression of PIF4, we sought to confirm that PIF4 protein expressed at endogenous levels displays similar temperature-dependent binding to the *FT* promoter. We therefore performed ChIP experiments on a *pif4-101* line complemented with *PIF4_{prom}::PIF4:ProteinA* (Supplementary Fig. 4). Consistent with the overexpression studies, we observed a strong increase in PIF4 binding to *FT* as a function of temperature. Reduced binding was observed at 17 °C, consistent with the very late flowering of plants under short days at low temperature, but this binding increased at 22 °C and was even higher at 27 °C (Fig. 3e). The temperature-dependent binding of PIF4 to *FT* could be due to growth temperature influencing the affinity of the PIF4 transcription factor for its binding site, or the efficiency of the ChIP could be affected by the temperature at which tissues were grown. To test these possibilities, we analysed another recently described PIF4 target locus¹⁸, *CYP79B2* (At4g39950), which is upregulated in 35S::*PIF4* (Supplementary Fig. 5a). We found PIF4 binding to occur constitutively at both 12 and 27 °C at a region in the first exon (Supplementary Fig. 5b). Another region further upstream in the promoter showed a temperature-dependent binding of PIF4, and, in both cases, no enrichment was seen for a control locus (Supplementary Fig. 5b). This indicates that the abundant PIF4 protein we observed at 12 °C is active and able to bind target sites, and confirms that the ChIP method per se is not influenced by the temperature at which the sample is grown, consistent with other studies¹⁹. The ability of PIF4 to bind loci in a more temperature-independent manner might explain why 35S::*PIF4* at 12 °C maintains hypocotyl and petiole elongation, while early flowering is strongly suppressed. We do not exclude that temperature may also influence PIF4 activity post-translationally.

Temperature signals are mediated through H2A.Z-nucleosomes in *Arabidopsis*²⁰, suggesting that temperature may be increasing the accessibility of the PIF4-binding site at the *FT* promoter. Consistent with this hypothesis, we found that H2A.Z-nucleosomes were present at the PIF4-binding site in the *FT* promoter. Furthermore, we found that the levels of H2A.Z-nucleosomes at the *FT* promoter decreased with higher temperature (Fig. 3f). These results suggest that the presence of H2A.Z-nucleosomes is limiting for PIF4 binding to *FT*, and that the PIF4 binding we observed at higher temperature is due to the greater accessibility of chromatin containing H2A.Z-nucleosomes at higher temperature. This suggests that in the absence of H2A.Z-nucleosomes, PIF4 should bind *FT* more strongly. We therefore compared the ability of PIF4 expressed under its own promoter to bind to the *FT* promoter in wild type and *arp6-1*, a background lacking incorporation of H2A.Z-nucleosomes. Interestingly, we observed considerably greater binding of PIF4 in *arp6-1* (Fig. 3g), indicating that H2A.Z-nucleosomes are rate limiting for PIF4 to activate *FT* expression. The eviction of H2A.Z-nucleosomes by higher temperature therefore provides a direct mechanism for the temperature-regulated expression of *FT* (Fig. 4c). Consistent with our previous results and the established role of H2A.Z in regulating temperature-dependent gene expression, we found that there is increased PIF4 messenger RNA in *arp6-1* background (Supplementary Fig. 6). However, our results for 35S::*PIF4* suppression by 12 °C indicate that transcriptional upregulation of PIF4 is not the rate-limiting step in regulating PIF4-mediated flowering at higher temperatures.

Our results indicate that the temperature-dependent regulation of *FT* by PIF4 is controlled at the level of chromatin accessibility of the

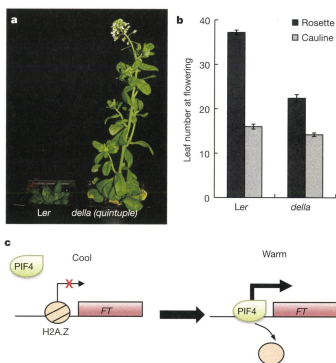


Figure 4 PIF4 integrates environmental signals. **a**, Suppression of flowering at 12 °C is significantly repressed in the absence of DELLA-mediated repression. **b**, Leaf number at flowering for *della* global is reduced compared with *Ler* at 12 °C (error bars, SD; $n = 6$). **c**, Representation of temperature-dependent *FT* regulation by PIF4. Temperature-induced H2A.Z nucleosome dynamics can regulate PIF4 binding to target loci for transcriptional activation.

FT promoter and possibly at the level of PIF4 protein activity. PIF4 activity is controlled through the repressive activity of DELLA proteins that prevent PIF4 binding DNA^{21,22}.

Consistently, plants having reduced or absent DELLA function are early flowering²³. We hypothesized that delay in flowering at lower temperatures might at least in part be due to DELLA-mediated repression of PIF4 activity. If so, it would be expected that absence of DELLAs should cause accelerated flowering at lower temperatures. In accord with this expectation, we found that a mutant lacking DELLAs flowered much earlier than wild type when grown at 12 °C (Fig. 4a, b). The phytohormone gibberellin triggers DELLA protein degradation, and plays a key permissive role for *FT* induction, because in a gibberellin-deficient background, gibberellin application increases *FT* expression 15-fold²⁴. Although it was proposed more than 50 years ago that gibberellins are upstream of florigen²⁵, the mechanism has not been clear. As DELLA proteins have been shown to be key regulators by which gibberellin influences PIF4, our finding that PIF4 is able to activate *FT* directly suggests a possible mechanism by which changes in gibberellin levels may influence flowering.

Climate change has already caused measurable changes in plant phenology and behaviour², and plants that incorporate temperature information into their life cycles appear to be able to adapt to warmer conditions more effectively than those that primarily rely on photoperiod to synchronize their lifestyles¹. The importance of the effects of climate change on yield are highlighted by the significant detrimental effects of increasing temperatures on yield⁴. PIF4 is a central integrator of environmental information in the plant and our finding that it activates *FT* at higher temperatures suggests it will be a key node for breeding crops resilient to climate change. This importance is suggested by the recent discovery that natural variation at *PIF4* plays a major role in key ecological traits²⁶.

METHODS SUMMARY

Detailed descriptions of the plant growth conditions, growth assays, transgenic constructs and ChIP techniques are provided in the Supplementary materials.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.V.K., D.L., K.E.J. and E.A. performed the experiments. N.P.H. and P.A.W. designed the study and supervised the work. All authors discussed the results and made substantial contributions to the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to P.A.W. (philip.wigge@sbcu.cam.ac.uk).

Role of corin in trophoblast invasion and uterine spiral artery remodelling in pregnancy

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In pregnancy, trophoblast invasion and uterine spiral artery remodelling are important for lowering maternal vascular resistance and increasing uteroplacental blood flow. Impaired spiral artery remodelling has been implicated in pre-eclampsia, a major complication of pregnancy, for a long time but the underlying mechanisms remain unclear^{1,2}. Corin (also known as atrial natriuretic peptide-converting enzyme) is a cardiac protease that activates atrial natriuretic peptide (ANP), a cardiac hormone that is important in regulating blood pressure³. Unexpectedly, corin expression was detected in the pregnant uterus⁴. Here we identify a new function of corin and ANP in promoting trophoblast invasion and spiral artery remodelling. We show that pregnant corin- or ANP-deficient mice developed high blood pressure and proteinuria, characteristics of pre-eclampsia. In these mice, trophoblast invasion and uterine spiral artery remodelling were markedly impaired. Consistent with this, the ANP potentially stimulated human trophoblasts in invading Matrigels. In patients with pre-eclampsia, uterine *Corin* messenger RNA and protein levels were significantly lower than that in normal pregnancies. Moreover, we have identified *Corin* gene mutations in pre-eclamptic patients, which decreased corin activity in processing pro-ANP. These results indicate that corin and ANP are essential for physiological changes at the maternal-fetal interface, suggesting that defects in corin and ANP function may contribute to pre-eclampsia.

Pregnancy poses a serious challenge for maintaining normal blood pressure. Pregnancy-induced hypertension, a major cause of maternal and fetal deaths, occurs in approximately 10% of pregnancies^{5,6}. During pregnancy, the uterus undergoes profound morphological changes, including trophoblast invasion and spiral artery remodelling. In pre-eclampsia, impaired spiral artery remodelling is common, but the underlying mechanisms are unclear^{2,7–9}. Studies indicate that vascular growth factor receptors, angiotensin and oestradiol are involved in the disease^{10–14}.

Corin is a cardiac protease that activates ANP, which is a cardiac hormone that regulates blood pressure and sodium homeostasis¹⁵. In mice, lack of CORIN prevents ANP generation and causes hypertension¹⁶. In humans, CORIN variants are associated with hypertension¹⁷. Interestingly, *Corin* expression was detected in the pregnant mouse⁴ (Fig. 1A) and human uterus (Supplementary Fig. 1). As a transmembrane protein, CORIN is expected to act at the expression sites, suggesting a possible function in the pregnant uterus.

To understand the role of CORIN in pregnancy, we created a mouse model in which a *Corin* transgene was expressed under a cardiac promoter (Fig. 1B). The transgenic and *Corin* knockout mice were crossed to generate mice expressing *Corin* only in the heart ('knockout/transgenic mice'; Fig. 1C, D). In knockout/transgenic mice, transgenic *Corin* expression restored pro-ANP processing in the heart (Supplementary Fig. 2) and normalized blood pressure (Fig. 1E),

indicating that cardiac CORIN was sufficient to maintain normal blood pressure in non-pregnant mice.

In pregnant *Corin* knockout mice, blood pressure increased at approximately 17 days post coitus and rose further before returning to the non-pregnant blood pressure level after delivery (Fig. 1F), which resembled late gestational hypertension in pre-eclamptic women. In *Corin* knockout/transgenic mice, which were normotensive, blood pressure increased similarly during pregnancy (Fig. 1G), indicating that cardiac *Corin* expression did not prevent pregnancy-induced hypertension. The data also show that in these mice, hypertension in pregnancy was not due to pre-existing high blood pressure. As well as in the uterus, *Corin* mRNA was detected in the umbilical cord and placenta (Supplementary Fig. 3). To distinguish the role of maternal *Corin* from that of placental or other fetal organs, *Corin* knockout females were mated with either wild-type or knockout males. The resulting fetuses carried one or no copy of the functional *Corin* gene. Normally, enzymes that are encoded by one gene copy are able to function. As shown in Fig. 1H, pregnant *Corin* knockout females that were mated with either wild-type or knockout males had similarly increased blood pressure, indicating that lack of maternal, but not fetal, *Corin* caused hypertension in pregnancy.

Proteinuria is a hallmark of pre-eclampsia. Wild-type, *Corin* knockout and knockout/transgenic mice had similar urinary protein levels before pregnancy and at mid gestation. However, the levels increased in *Corin* knockout and knockout/transgenic mice at late gestation (Fig. 1I), consistent with reported proteinuria in mouse models of pre-eclampsia¹⁸. Ischaemic glomeruli, indicated by fewer red blood cells, were found in pregnant *Corin* knockout and knockout/transgenic mice (Fig. 1J, a–f) but not in non-pregnant mice (Supplementary Fig. 4). Periodic acid–Schiff staining revealed increased extracellular matrices and collapsed glomerular capillaries in pregnant *Corin* knockout and knockout/transgenic mice (Fig. 1J, g–i). Electron microscopy showed narrow glomerular capillary lumens and thick basement membranes (Fig. 1K), suggesting endotheliosis and increased extracellular matrices. Additional pathological features such as necrotic cells and calcium deposits in the placental labyrinth also existed in these mice (Supplementary Fig. 5), indicating insufficient uteroplacental perfusion. Consistent with this, *Corin* knockout and knockout/transgenic mice had smaller litters (7.1 ± 2.3 (*n* = 28) and 6.8 ± 2.7 (*n* = 28) pups per litter, respectively, versus wild-type mice, which had 9.1 ± 1.2 (*n* = 21) pups per litter; *P* < 0.001 in both cases).

We examined embryos at embryonic day 12.5 (E12.5), an early time point before blood pressure increase in *Corin* knockout and knockout/transgenic mice, and E18.5 (two days before delivery). Wild-type E12.5 embryos showed obvious trophoblast invasion, shown by cytokeratin staining (Fig. 2A), and large vessels mostly in the deep decida, shown by smooth-muscle α -actin (SMA) staining (Fig. 2B), indicating that

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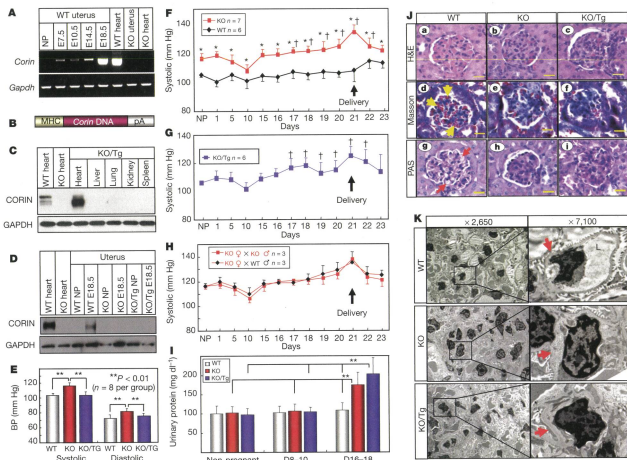


Figure 1 | Hypertension, proteinuria and renal pathology in pregnant *Corin* knockout and knockout/transgenic mice. **A**, *Corin* mRNA expression in mouse uteri. **B**, *Corin* transgenic (Tg) construct. **C**, **D**, Western blot analysis of *CORIN* protein in wild-type (WT), *Corin* knockout (KO) and knockout/transgenic mice. **E**, Blood pressure (BP, mean $\pm$ s.d.) in non-pregnant females. **F**, **G**, Blood pressure increased in *Corin* knockout (F) and knockout/transgenic (G) mice in pregnancy. Data are mean $\pm$ s.d. * P < 0.05 versus WT of the same time point. ** P < 0.05 versus non-pregnant level of the same genotype. **H**, Similar blood-pressure changes in *Corin* knockout females mated with knockout or WT males. **I**, Late gestational proteinuria in *Corin*

knockout and knockout/transgenic mice. Data are mean $\pm$ s.d. ** P < 0.01, n = 7 or 8 per group. **J**, **a–i**, Renal ischaemia in pregnant *Corin* knockout and knockout/transgenic mice. E18.5 sections are stained with haematoxylin and eosin (H&E), Masson trichrome or periodic acid–Schiff (PAS). Scale bar, 20 μ m. Red blood cells (yellow arrows) and open capillaries (red arrows) in WT glomeruli are shown. **K**, Narrow glomerular capillary lumen (L) and thick basement membranes (red arrows) in *Corin* knockout and knockout/transgenic mice at E18.5 shown by electron microscopy. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NP, non pregnant; pA, poly A.

smooth muscles in the superficial decidua were replaced by invading trophoblasts. In contrast, trophoblast invasion in *Corin* knockout and knockout/transgenic embryos was markedly reduced (Fig. 2a) and smaller arteries were found in both superficial and deep decidua (Fig. 2b). In E18.5 wild-type embryos, more abundant trophoblasts were found in the decidua and myometrium compared with those in *Corin* knockout and knockout/transgenic mice (Fig. 2c, d). By haematoxylin and eosin staining, larger and more abundant decidua spiral arteries were observed in wild-type than in *Corin* knockout or knockout/transgenic mice (Fig. 2e). Figure 2f–h shows strong cytokeratin (trophoblasts) staining but weak von Willebrand factor (endothelial) and SMA (smooth muscle) staining in wild-type decidua and myometrial arteries. These data indicate that trophoblast invasion and spiral artery remodelling were impaired in *Corin* knockout and knockout/transgenic mice, and that this defect occurred before blood pressure increased in these mice.

CORIN activates ANP in the heart¹⁵ but it was unknown whether the *CORIN* function in pregnancy was also mediated by ANP. Pro-ANP is expressed in the non-pregnant and pregnant uterus (Supplementary Fig. 6). If *CORIN* acts on pro-ANP to promote trophoblast invasion and spiral artery remodelling, thereby preventing hypertension in pregnancy, ANP (also known as *Nppa*) and *Corin* knockout mice should have similar phenotypes. ANP knockout mice are hypertensive (Fig. 3a)

but their blood pressure was not monitored during pregnancy¹⁹. We found similarly increased blood pressure in pregnant ANP knockout mice (Fig. 3b). The mice also had late gestational proteinuria (Fig. 3c) and smaller litters (4.4 $\pm$ 1.7 (n = 25) versus wild-type, 9.1 $\pm$ 1.2 (n = 21) pups per litter, P < 0.001). By immunostaining, impaired trophoblast invasion and smaller spiral arteries were observed in E12.5 embryos (Fig. 3d, e). In E18.5 embryos, ANP knockout mice had far fewer trophoblasts (Fig. 3f, g) and smaller arteries (Fig. 3h) in the decidua and myometrium than those in wild-type mice. Consistent with this, weak cytokeratin-staining but strong von Willebrand factor-staining were found in arteries in ANP knockout mice (Fig. 3i). Thus, ANP and *Corin* knockout mice had very similar phenotypes, indicating that the role of *CORIN* in pregnancy is probably mediated by ANP.

In the heart, *CORIN* produces ANP, which then regulates blood pressure by promoting natriuresis and vasodilation⁵. Here we found that lack of *CORIN* and ANP impaired trophoblast invasion and spiral artery remodelling, which was not rescued by cardiac *Corin* expression in *Corin* knockout/transgenic mice. ANP is known to relax vascular smooth muscles. Recently, ANP and its downstream cyclic GMP-dependent protein kinase were shown to be important in angiogenic processes by promoting endothelial regeneration^{20,21}. Thus, ANP may function locally to remodel uterine arteries. Our results also indicate that ANP may directly promote trophoblast invasion (Fig. 4a), and we

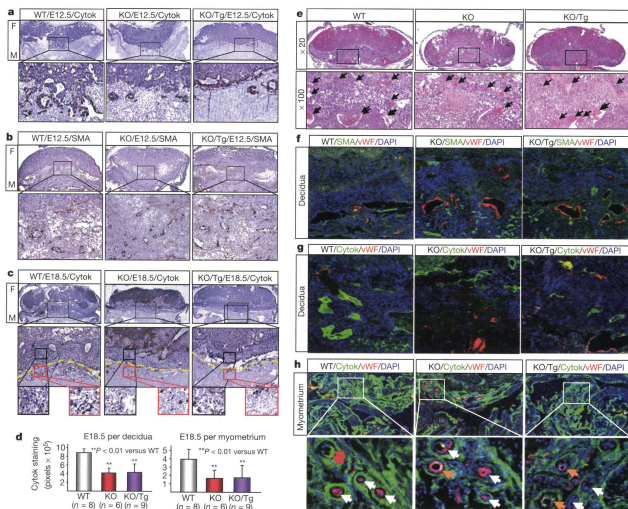


Figure 2 | Impaired trophoblast invasion and spiral artery remodelling in *Corin* knockout and knockdown/transgenic mice. a, b, E12.5 embryos were stained for trophoblasts (a) or smooth muscles (b). Fetal (F) and maternal (M) sides are indicated. Boxed areas in top panels are shown at a higher magnification ($\times 200$). c, E18.5 embryo sections were stained for trophoblasts. In lower panels ($\times 100$), yellow lines show the decidua and myometrium boundary.

therefore tested this idea. We found that ANP markedly stimulated human trophoblasts to invade Matrigels (Fig. 4b) (Supplementary Fig. 7a). In these cells, ANP receptor (also known as atrial natriuretic peptide receptor 1) mRNA expression was confirmed (Supplementary Fig. 7b) and ANP-stimulated intracellular cGMP production was detected (Fig. 4c) (Supplementary Fig. 7c).

Our findings emphasize the importance of local ANP production by CORIN, which acts on trophoblasts and vascular cells in the uterus. Because heart-derived ANP circulates inside the vessel, our model may explain why cardiac CORIN failed to promote trophoblast invasion and uterine artery remodelling, as shown in *Corin* knockout/transgenic mice. To verify this hypothesis, we quantified *Corin* mRNA and protein in human uteruses by polymerase chain reaction with reverse transcription (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). The levels were low in non-pregnant women but increased in pregnant women (Fig. 4d, e). In pre-eclamptic women, the levels were significantly lower than in normal pregnancies. Similar results were found by immunostaining (Fig. 4d and Supplementary Fig. 8). Consistent with this, pro-ANP levels in uterine tissues were significantly higher in pre-eclamptic women than in normal pregnant women (Fig. 4f), indicating that reduced uterine *Corin* expression impaired pro-ANP processing in these patients. *Corin* is a membrane-bound protein⁴¹⁵, and recent studies showed that CORIN can be shed from

d. Quantitative data (mean $\pm$ s.d.) of cyokeratin (Cytok) staining. e, Fewer and smaller decidual spiral arteries (arrows) in H&E-stained E18.5 *Corin* knockout and knockdown/transgenic embryos. f–h, Co-staining of SMA, von Willebrand factor (vWF), cyokeratin and nuclei in E18.5 embryos. Red arrows indicate cyokeratin (green) signals, white arrows indicate von Willebrand factor (red) signals and orange arrows indicate mixed (yellow) signals.

cardiomyocytes and that soluble CORIN is found in human plasma^{22,23}. We found that plasma CORIN levels were higher in pre-eclamptic patients than non-pregnant or normal pregnant women (Fig. 4g). Thus, CORIN levels in plasma did not reflect the levels in tissues, indicating that plasma CORIN was probably derived from the heart, where *Corin* expression increased in response to high blood volume and high blood pressure in pregnancy. These results provide further support for a local function of CORIN in the pregnant uterus.

We next sequenced the *CORIN* gene²⁴ in pre-eclamptic patients and identified a mutation that alters Lys to Glu at position 317 in low-density lipoprotein receptor repeat 2 in one woman (Fig. 4h, j) and another mutation altering Ser to Gly at position 472 in the frizzled 2 domain in two women from the same family who had pre-eclampsia (Fig. 4i, j). In functional studies, Lys317Glu and Ser472Gly mutations did not affect CORIN expression in HEK293 cells but markedly reduced CORIN activity in processing pro-ANP (Fig. 4k–n). The data were consistent with previous findings that low-density lipoprotein receptor repeats and frizzled domains are critical for CORIN activity²⁵, suggesting that the mutations may impair CORIN function in the patients, thereby contributing to pre-eclampsia. Interestingly, CORIN variants in the frizzled 2 domain that impaired CORIN function have been reported in African American people^{17,26}, a high-risk population for pre-eclampsia.

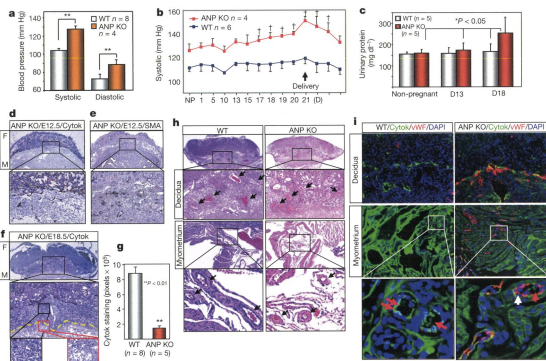


Figure 3 | Hypertension, proteinuria and uteroplacental pathology in pregnant ANP knockout mice. **a**, Blood pressure (mean $\pm$ s.d.) in non-pregnant females. $^{**}P < 0.01$. **b**, Elevated blood pressure (mean $\pm$ s.d.) in pregnant ANP knockout mice. $^{*}P < 0.05$ versus non-pregnant level. **c**, Gestational proteinuria in ANP knockout mice. Data are mean $\pm$ s.d. **d**, **e**, Impaired trophoblast invasion and smooth muscle remodelling in E12.5 embryos stained for cytokeratin (**d**) or SMA (**e**). Boxed areas in top panels are

shown at a higher magnification ($\times 200$). **f**, Impaired trophoblast invasion in E18.5 embryos stained for cytokeratin. **g**, Quantitative data (mean $\pm$ s.d.) of cytokeratin staining in E18.5 ANP knockout embryos. **h**, Impaired decidua and myometrial artery remodelling (arrows) in H&E-stained E18.5 ANP knockout embryos. **i**, Co-staining of cytokeratin, von Willebrand factor and nuclei in E18.5 ANP knockout embryos. Red arrows indicate cytokeratin (green) signals and white arrows indicate von Willebrand factor (red) signals.

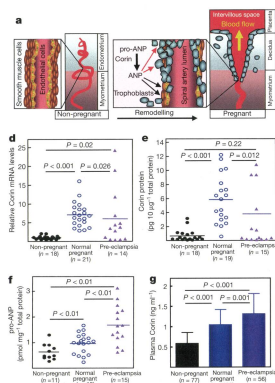


Figure 4 | ANP-stimulated human trophoblast invasion, and impaired uterine Corin expression and Corin mutations in pre-eclamptic patients. **a**, A model showing that CORIN-produced ANP in the pregnant uterus promotes

trophoblast invasion (red arrows) and vascular-wall remodelling (black arrows). **b**, **c**, ANP-stimulated human BeWo trophoblasts and primary trophoblasts in Matrigel invasion (**b**) and intracellular cGMP production (**c**). Data are mean $\pm$ s.d. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control. **d**–**f**, Corin mRNA (**d**) and protein (**e**), and pro-ANP levels (**f**) in human uterus samples. Horizontal lines indicate mean values. **g**, Plasma-soluble CORIN levels (mean $\pm$ s.d.) in pre-eclamptic patients and normal controls. **h**–**j**, CORIN gene mutations causing Lys317Glu (**h**) and Ser472Gly (**i**) changes in CORIN (**j**). **k**, **l**, Expression of Lys317Glu and Ser472Gly mutants in HEK293 cells (top panels). Vector, WT CORIN and inactive CORIN Arg801Ala and Ser985Ala mutants were controls. Lys317Glu and Ser472Gly mutations reduced pro-ANP processing activity (bottom panels). **m**, **n**, Quantitative data (mean $\pm$ s.d.) from three experiments or more. Fz, frizzled; LDLR, LDL receptor; SR, scavenger receptor; TM, transmembrane.

Previously, high levels of plasma pro-ANP or ANP were detected in pre-eclamptic patients^{27,28}. As shown by our plasma soluble CORIN data, plasma protein levels may not reflect those in tissues. Taken together, our data show a novel local function of CORIN and ANP in promoting trophoblast invasion and spiral artery remodelling to prevent hypertension in pregnancy. The data suggest that impaired *Corin* expression or function in the pregnant uterus may be an important mechanism underlying pre-eclampsia. Studies to better understand impaired uterine *Corin* expression in pre-eclamptic patients may help to develop new strategies to enhance the CORIN-ANP pathway and prevent or treat this life-threatening disease.

METHODS SUMMARY

Corin and ANP knockout mice have been described previously^{16,19}. Transgenic mice with cardiac *Corin* expression were generated using a heart-specific promoter. Blood pressure was measured by radiotelemetry¹⁶. Tissue sections from non-pregnant and pregnant mice were stained with haematoxylin and eosin, Masson's trichrome, periodic acid-Schiff or von Kossa, or immunostained with antibodies against cytokeratin, SMA, von Willebrand factor or CORIN. Renal sections were also examined by electron microscopy. Trans-well invasion assay was carried out with human primary villous trophoblasts (ScienCell) and trophoblastic JEG3, BeWo, JAR cell lines (ATCC) in Matrigel Invasion Chambers (BD Biosciences). ANP-stimulated cGMP production in trophoblasts was assayed in 96-well plates. Intracellular cGMP levels were determined using an enzyme immunoassay kit (Enzo Life Sciences). *Corin* levels in human blood and uterus tissue samples were measured using ELISA²². Pro-ANP levels in human uterus tissues were also measured using ELISA (Alpco Diagnostics). *Corin* gene exons²⁴ from pre-eclamptic patients were PCR-amplified and sequenced directly. *Corin* gene mutations that were identified were studied by expressing mutant CORIN proteins in HEK293 cells and testing their activities in pro-ANP processing assays, as described previously²⁵.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions Y.C., W.W., N.D., J.L., D.K.S., M.L., C.F., J.P., S.C., S.W., Z.L. and L.D. designed and performed experiments. N.D., W.C. and X.H. collected patient samples and analysed clinical data. Q.W. conceived the study and designed experiments. Y.Z. and Q.W. wrote the manuscript. All authors analysed and interpreted data, and critically read the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to Q.W. (wuq@ccf.org).

Genome-wide protein–DNA binding dynamics suggest a molecular clutch for transcription factor function

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Dynamic access to genetic information is central to organismal development and environmental response. Consequently, genomic processes must be regulated by mechanisms that alter genome function relatively rapidly^{1–4}. Conventional chromatin immunoprecipitation (ChIP) experiments measure transcription factor occupancy⁵, but give no indication of kinetics and are poor predictors of transcription factor function at a given locus. To measure transcription-factor-binding dynamics across the genome, we performed competition ChIP (refs 6, 7) with a sequence-specific *Saccharomyces cerevisiae* transcription factor, Rap1 (ref. 8). Rap1-binding dynamics and Rap1 occupancy were only weakly correlated ($R^2 = 0.14$), but binding dynamics were more strongly linked to function than occupancy. Long Rap1 residence was coupled to transcriptional activation, whereas fast binding turnover, which we refer to as ‘treadmilling’, was linked to low transcriptional output. Thus, DNA-binding events that seem identical by conventional ChIP may have different underlying modes of interaction that lead to opposing functional outcomes. We propose that transcription factor binding turnover is a major point of regulation in determining the functional consequences of transcription factor binding, and is mediated mainly by control of competition between transcription factors and nucleosomes. Our model predicts a clutch-like mechanism that rapidly engages a treadmilling transcription factor into a stable binding state, or vice versa, to modulate transcription factor function.

The diverse biological functions of Rap1 (ref. 9) make it an excellent model for testing the hypothesis that binding dynamics are important for transcription factor function (Supplementary Fig. 1). We developed a strain with two copies of *RAP1*. One copy of *RAP1* was tagged with a 3× Flag epitope and was constitutively expressed from the endogenous *RAP1* promoter. A second copy of *RAP1* was tagged with a 9× Myc epitope and was controlled by a weakened galactose-inducible promoter, *GALL* (an attenuated version of the *GALI* promoter) (Fig. 1a). This strain showed no growth defects in inducing (2% galactose) or non-inducing (2% dextrose) conditions (Fig. 1b and Supplementary Fig. 2). To avoid cell-cycle and DNA replication effects, for the duration of the experiment the strain was arrested in G1 with alpha factor⁶. The induced Rap1 protein isoform could be detected as early as 30 min after galactose induction (Fig. 1c). The ratio of Rap1 isoforms provided an estimate of the nucleoplasmic pool of Rap1 molecules (Fig. 1d). We then performed Myc and Flag ChIP experiments independently from extract corresponding to each of 10 time points (0, 10, 20, 30, 40, 50, 60, 90, 120 and 150 min after induction). We also performed ChIP to measure total DNA occupancy using a Rap1-specific antibody at 0 and 60 min. DNA fragments enriched in the

ChIPs were detected on whole-genome-tiling 12-plex microarrays containing 270,000 probes per subarray, with an average probe interval of 41 bp and an average probe length of 54 bp (Supplementary Fig. 3). The entire time-course experiment was performed in duplicate. (Procedural details can be found in the Methods.)

After induction, Rap1–Myc was incorporated at targets where Rap1 had previously been shown to bind^{8,10} (Fig. 2a, b), indicating that the system was functioning as designed. The increase in Rap1 protein caused by the induction of the competitor did not cause an increase in the overall occupancy at the measured Rap1 sites (Fig. 2c, d and Supplementary Figs 4 and 5). As Rap1–Myc ChIP occupancy increased at sites of Rap1 binding, Rap1–Flag occupancy decreased coordinately (Fig. 2c, d and Supplementary Fig. 4). Thus, Rap1–Myc is competing specifically with Rap1–Flag at each locus, and Rap1–Myc binding is not the result of cooperativity or additional Rap1 binding locations.

To interpret our data, we developed a model to determine turnover rates of Rap1 by modifying a fitting algorithm used previously to

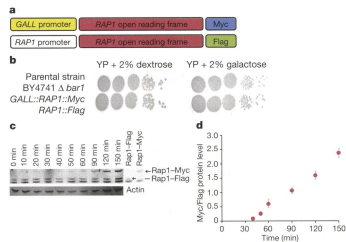


Figure 1 | Development of transcription factor competition ChIP yeast strains. **a**, Schematic of the Rap1 competition-ChIP yeast strain. **b**, Growth comparison of competition yeast strain and wild-type in inducing (2% galactose) and non-inducing (2% dextrose) conditions. **c**, Western blot analysis using an antibody against Rap1 (y-300). Strains containing only a Rap1–Myc or only Rap1–Flag copy are shown to the right to indicate the size of isoform-specific bands. The actin loading control is shown below. **d**, To estimate the dynamics of induction, the ratio of induced Rap1–Myc and constitutive Rap1–Flag protein is plotted. Data are from two technical replicates of two independent time-course replicates. Error bars represent s.e.m.

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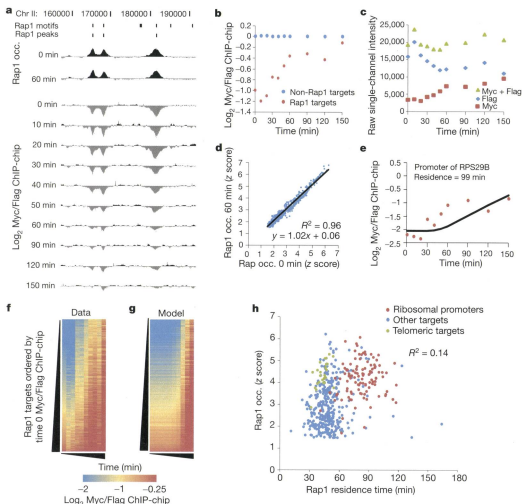


Figure 2 | Rap1-bound sites exhibit distinct replacement dynamics. **a**, A Rap1 turnover experiment over a 30-kb region of chromosome II (Chr II). Rap1 motifs and peaks are shown. **b**, Average log₂ Myc/Flag ratio values for all Rap1 targets (red) increase relative to non-Rap1 targets (blue). **c**, Rap1–Myc competes with Rap1–Flag for binding. Average single-channel intensity for Rap1–Myc and Rap1–Flag for a single probe (CHR15FS000978891) in the promoter of the *TYE7* gene shows that the increase in Rap1–Myc is coincident with the loss of Rap1–Flag. **d**, Total Rap1 occupancy does not change during the time course. Average total Rap1 occupancy (occ.) (log₂ ratio of Rap1 immunoprecipitation

(y-300) to input ratio z score) at Rap1 targets at time 0 is plotted against that at 60 min. **e**, Average log₂ Myc/Flag ratios for the promoter of ribosomal protein gene *RPL29B* (red points). The model fit for the residence time parameter that best fits these data is shown (black line). **f**, Colorimetric representation of log₂ Myc/Flag ratios for all 465 Rap1 targets, sorted by the initial (normalized) log₂ Myc/Flag ratio. **g**, For each site in **f**, the log₂ Myc/Flag ratio predicted by our residence time model, based on the calculated residence time, is shown. **h**, Rap1 occupancy (time 0 z score) versus Rap1 residence for 465 Rap1 targets ($R^2 = 0.14$, 0.37; Spearman rank correlation).

measure histone H3 turnover⁶. Under our experimental conditions, the extracted turnover rate for a transcription factor at a binding site is equivalent to its dissociation rate, which allows us to measure the residence time (Supplementary Figs 6–8 and Supplementary Text). Our experimental system can quantify binding events that have an apparent duration of 500 s or longer (Supplementary Figs 6 and 7, and Supplementary Text). Using our ChIP data and model we measured residence time of Rap1 at 439 peaks of Rap1 enrichment genome-wide, and at the 26 uniquely mappable telomeres (Fig. 2e–h, Supplementary Fig. 9 and Supplementary Text). Rap1 occupancy correlated only modestly with Rap1 residence ($R^2 = 0.14$; Spearman rank correlation = 0.37) (Fig. 2h), and distinct dynamics of Rap1–Myc incorporation were observed at different genomic loci (Fig. 2e–h). Thus, residence time and occupancy are distinct measurements, and our system was capable of distinguishing Rap1 turnover kinetics at different loci in the same experiment.

We found that efficient transcriptional activation was associated with stable Rap1 binding, whereas lower transcript production was associated with treadmilling, despite similar levels of Rap1 occupancy. Long Rap1 residence times occurred at ribosomal protein gene

promoters, which are very highly transcribed and strongly activated by Rap1 (refs 6, 11, 12) (Fig. 2e, h). In contrast, Rap1 binding to non-ribosomal protein targets and to the infrequently transcribed telomeric and subtelomeric Rap1 sites was characterized by fast turnover (Fig. 2h and Supplementary Figs 10 and 11). Stable Rap1 binding seems to support higher mRNA production through more efficient recruitment of the RNA polymerase II machinery¹² (Fig. 3a). Genes with stable Rap1 binding at their promoters indeed exhibited high levels of RNA polymerase II association⁷ (Fig. 3a), high transcription initiation rates^{11,13} (Fig. 3b) and high mRNA levels (Fig. 3c). Rap1 occupancy does not correspond as strongly (Fig. 3a–c; right panels). TATA-binding protein (TBP) turnover⁷ is also slow at ribosomal protein genes, indicating that slow transcription-factor-binding dynamics may be a hallmark of efficient transcription initiation^{11,13}.

We next examined possible mechanisms for the locus-specific differences in Rap1 residence time. Nucleosomes are a major regulator of genome accessibility¹⁴, so we examined the relationship between histone modification and Rap1-binding dynamics^{15,16}. Sites of long Rap1 residence were strongly correlated with sites of enrichment for the histone acetyltransferases Gcn5 and particularly Esa1 (ref. 17) (Fig. 3d, e).

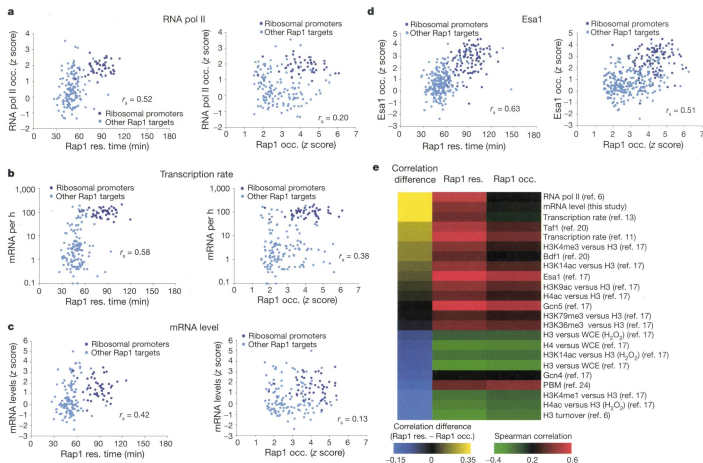


Figure 3 | RNA polymerase II recruitment, mRNA production, and histone acetyltransferase recruitment is associated with long Rap1 residence.

a–d, Left, Rap1 residence (res.) time is plotted on the x axis. Right, Rap1 occupancy is plotted on the x axis. In both panels, the following are plotted on the y axis: RNA polymerase II (RNA pol II) occupancy (**a**); the number of mRNA transcripts per h (**b**) (ref. 11); mRNA levels at time 0 (**c**); and histone acetyltransferase Esal occupancy z scores (**d**)¹⁷. r_s is the Spearman correlation

Nucleosome instability reinforced by Gcn5 and Esa1 (members of SAGA and NuA4, respectively) may stabilize Rap1 binding by reducing competition with nucleosomes^{18,19}. Other indicators of active promoters—including H3K4me3, occupancy by the bromodomain protein Bdf1 (similar to mammalian TAF1)²⁰, and acetylation of H3K9, H4 and H3K14—were also more strongly associated with Rap1 residence time than with Rap1 occupancy (Fig. 3e).

In general, sites that are bound by Rap1 are strongly depleted of nucleosomes²¹. However, the binding dynamics data allowed us to appreciate a more complex relationship. We grouped Rap1-bound loci into four categories based on their measured Rap1 residence time: longest, long, short, and shortest. We then aligned the Rap1 motifs in each category and plotted nucleosome occupancy relative to the motif position, reasoning that nucleosomes in direct proximity to the DNA motif bound by Rap1 would have a strong influence on Rap1 residence times²¹. As expected, strong nucleosome depletion was centred on the Rap1 motif (Fig. 4a). However, as Rap1-binding turnover increased, nucleosome depletion was correspondingly less pronounced. Thus, not all highly occupied Rap1 sites are equally depleted of nucleosomes *in vivo*. Instead, a subset of loci at which Rap1 occupancy is high but binding turnover is also high (treadmilling) are associated with higher nucleosome occupancy (Supplementary Fig. 11b). No consistent relationship is apparent when Rap1 targets are grouped by occupancy, as measured by traditional ChIP (Fig. 4a).

We next examined nucleosome occupancy on naked DNA in the absence of Rap1 or any protein cofactors²¹. Notably, DNA-encoded

value. **e**, Colorimetric representation of the Spearman rank correlation between various genomic data sets and Rap1 occupancy (right) and Rap1 residence (left), ordered by the magnitude of the difference between the residence and occupancy correlations for each comparison (left). WCE, whole-cell extract; PBM, protein-binding microarray. Telomeric targets are excluded from analysis (Supplementary Text).

nucleosome occupancy measured *in vitro* is low only for the class of Rap1 targets with the most stable binding (Fig. 4b and Supplementary Fig. 11b). This pattern was not recapitulated when Rap1 targets were sorted by occupancy (Fig. 4b). This suggests that the nucleosome behaviour surrounding transcription factor motifs is at least partially encoded in DNA¹⁵, and that this DNA-encoded nucleosome occupancy can influence the binding dynamics of transcription factors, and thereby affect functional outputs (Supplementary Fig. 11a–c).

We sought further evidence supporting direct competition between nucleosomes and Rap1. We compared histone H3 turnover⁶ to Rap1 residence times and found that loci with long Rap1 residence times also had relatively slow H3 turnover. Similarly, histone H3 molecules that treadmill are found almost exclusively at sites of Rap1 treadmill (Fig. 4c). Rap1–nucleosome interactions isolated by immunoprecipitating Rap1 after MNase digestion²² were also detected more often at treadmill sites (Fig. 4d). Further evidence for competition is supported by a marked increase in nucleosome occupancy directly over Rap1 motifs after Rap1 depletion²³ at treadmill loci, but not at loci with stable Rap1 binding (Fig. 4e). These relationships provide evidence for direct competition between Rap1 and nucleosomes.

Given that high DNA-encoded nucleosome occupancy is associated with rapid Rap1 turnover (Fig. 4b), it is reasonable to expect that differences in the strength of the DNA motif bound by the transcription factor would also influence turnover. To test this, we examined the relationship between Rap1 turnover and experimentally measured *in vitro* Rap1 affinity at each locus²⁴. For sites with longer Rap1 residence,

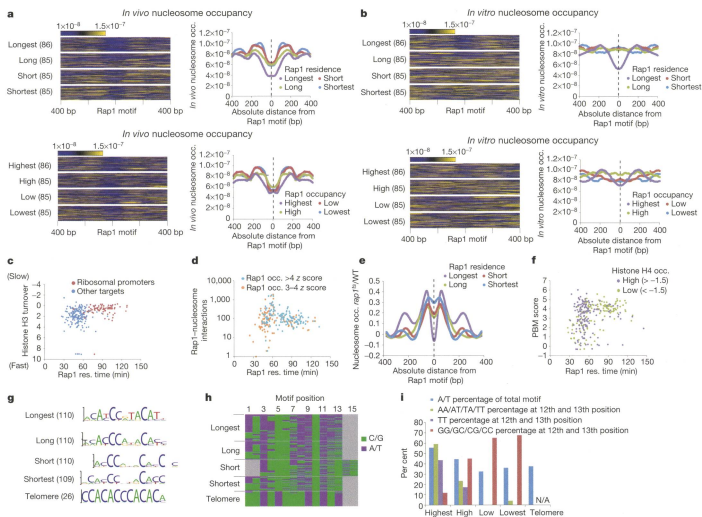


Figure 4 | Evidence for competition between Rap1 and nucleosomes.

a, Colorimetric representation of *in vivo* nucleosome occupancy centred on Rap1-binding motifs. Loci are ordered by Rap1 residence time (top) or Rap1 occupancy (bottom). The total number of Rap1 targets in each group is shown in parentheses. To the right are plots of the average nucleosome occupancy for each group centred on the Rap1 motif. Targets with several Rap1 motifs are represented by one randomly chosen motif. **b**, Same as **a** but for *in vitro* nucleosome occupancy. **c**, Histone H4 turnover versus Rap1 residence for ribosomal protein genes (red) and other targets (blue). **d**, The number of Rap1–nucleosome interactions²⁷ detected within each Rap1 target peak boundary on a log₁₀ scale. **e**, Relative change in nucleosome occupancy following Rap1 depletion²³ centred on Rap1 motifs grouped by residence time. A value of zero

represents no relative change in nucleosome occupancy. **f**, *In vitro* Rap1 affinity for its cognate target, as measured using a PBM¹⁴, compared to Rap1 residence. Colours represent histone H4 occupancy z scores (> -1.5 purple (high), < -1.5 green (low))¹⁷. **g**, Top-position weight matrix motifs discovered for Rap1 targets, grouped by residence. The number of targets for each group is in parentheses. **h**, All motifs from the top-position weight matrix for each residence group are coloured by their A or T (A/T) (purple), or G or C (G/C) (green) content at each base position. **i**, Percentage of A/T content for the entire motif (blue), AA/AT/TA/TT at the twelfth and thirteenth motif position (green), TT at the twelfth and thirteenth position (purple) and GG/GC/CG/CC at the twelfth and thirteenth position (red) for Rap1 targets, grouped by residence and telomeric regions.

Rap1's affinity for DNA was generally high, whereas Rap1 sites with the fastest turnover had lower experimentally measured Rap1 affinity (Fig. 4f). Despite this relationship, among sites with strong Rap1 motifs, nucleosome occupancy was still the major factor distinguishing sites with long Rap1 residence times from those with higher turnover (Fig. 4f).

Longer *in vivo* Rap1 residence times at sites of high Rap1 affinity are consistent with control of the Rap1–nucleosome competition being encoded directly in the DNA sequence to a substantial degree^{15,16}. We reasoned that this would be reflected in the sequence of the DNA motifs bound by Rap1. Indeed, we found differences in the composition of the Rap1 motifs for each of the turnover categories, with the longest residence Rap1 sites preferentially containing A or T at positions 4, 8, 12 and 13 (Fig. 4g–i). These associations were not as strong when Rap1 targets were ordered by occupancy (Supplementary Fig. 12). Sites at which residence was shortest tended to contain a degenerate Rap1 binding motif (Fig. 4g).

For several other transcription factors, microscopy-based measurements at individual loci point to much shorter residence times than those measured for Rap1 (refs 1, 3, 4, 25–27). For example, despite an *in vitro* residence time similar to Rap1 (~90 min^{9,28}), glucocorticoid-receptor binding seems to be exceptionally short-lived at individual loci^{26,27}. Nevertheless, an overall positive relationship between residence time and transcriptional output is observed for both Rap1 and glucocorticoid receptor^{12,29}. The differences in Rap1 and glucocorticoid-binding dynamics, and the disparity between glucocorticoid-receptor residence time *in vitro* and *in vivo*, may reflect different modes of interactions with nucleosomes. The binding affinity of glucocorticoid receptor may be particularly sensitive to nucleosome packaging or may be regulated by the availability of DNA that is transiently accessible from the nucleosome surface¹⁴. This type of accessibility on the nucleosome itself could be regulated, and would not rely on the complete loss of a nucleosome¹⁴. Rap1 itself exhibits such properties, with its binding progressively inhibited as the motif recognized by

Rap1 is moved closer to the nucleosome dyad³⁰. Our data do not exclude a model in which transcription factor binding occurs adjacent to a nucleosome, and competition occurs without complete nucleosome eviction^{1,4,22,30}.

In this study, we determined Rap1-binding dynamics genome-wide using competition ChIP. Rap1 occupancy was only weakly correlated with Rap1 binding turnover, showing that these are independently measurable properties. Binding turnover correlates more strongly than occupancy with many aspects of genomic function, most predominantly RNA polymerase II recruitment and transcript levels. Stable Rap1 binding is associated with activation, whereas Rap1 treadmilling is associated with higher nucleosome occupancy, nucleosomal treadmilling, and a lack of transcription. Our work provides the basis for a model in which transcription-factor-binding dynamics is a major point of regulation in determining the functional consequences of transcription factor binding. Importantly, this model provides a plausible mechanism for a locus-specific switch between inactive and active transcription factor states, or even for a rapid switch from an activator (stable binding) to a repressor (treadmilling), or vice versa. This could be achieved at any given locus through a 'clutch' that alters the balance of the continual competition between transcription factors and nucleosomes (Supplementary Fig. 1). This clutch could operate through histone modification, histone-variant incorporation, ATP-dependent chromatin remodelling, cofactor binding, or any other site-directed chromatin altering activity.

METHODS SUMMARY

Strain construction. The *RAP1* gene and promoter was cloned into the pRS403 plasmid and integrated by homologous recombination into the *HIS3* locus of the BY4741 *S. cerevisiae* strain. The two copies of *RAP1* were then sequentially tagged using the 9× Myc epitope from pYM20hphNT1 at the *HIS3* copy of *RAP1* and the 3× Flag tag from p3Flag-KanMX at the endogenous *RAP1* copy. The *HIS3* copy of the *RAP1* promoter was replaced using homologous recombination by amplifying the *GALL-natNT2* promoter from the pYM-N27 plasmid. Integrations were confirmed using PCR and western blot analysis. The *RAP1* gene was knocked out by homologous recombination using a *LEU2* gene amplified from pRS405.

Time course. Yeast were grown overnight in YPD (yeast extract 1%, peptone 2% and dextrose 2%) to an inoculate at 600 nm (D_{600}) of 0.2 (Genesys 20 Spectrophotometer) in a 4-l Erlenmeyer flask. These cells were grown to a D_{600} of 0.4 and subsequently arrested using 5 μM alpha factor (400 μl of 10 mM, GenScript) until 95% of the yeast cells were unbudded (~3 h). Cells were then induced by adding 40% galactose to a final concentration of 2%. At this time, additional alpha factor was added (400 μl of 10 mM, GenScript). Samples were collected at time points 0, 10, 20, 30, 40, 50, 60, 90, 120 and 150 min after galactose induction. At each time point, 35 ml of culture was taken and added immediately to 37% formaldehyde to a final concentration of 1% for 20 min. Thirteen millilitres were taken for subsequent RNA preparation. Two millilitres were taken for protein preparation by pelleting cells and heating at 95 °C for 5 min in 0.6 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.0025% bromophenol blue. All samples were frozen immediately in liquid nitrogen.

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Author Information Data has been deposited in the Gene Expression Omnibus under accession numbers GSE32351 (ChIP-on-chip data), GPL14612 (ChIP platform), GSM677030-GSM677033 (RNA expression array data) and GPL4414 (expression platform). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on this article online using the article www.nature.com/nature. Correspondence and requests for materials should be addressed to J.D.L. (jlleb@bio.unc.edu).

A new understanding of the decoding principle on the ribosome

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During protein synthesis, the ribosome accurately selects transfer RNAs (tRNAs) in accordance with the messenger RNA (mRNA) triplet in the decoding centre. tRNA selection is initiated by elongation factor Tu, which delivers tRNA to the aminoacyl tRNA-binding site (A site) and hydrolyses GTP upon establishing codon–anticodon interactions in the decoding centre^{1–9}. At the following proofreading step the ribosome re-examines the tRNA and rejects it if it does not match the A codon^{2,3,10–14}. It was suggested that universally conserved G530, A1492 and A1493 of 16S ribosomal RNA, critical for tRNA binding in the A site^{15–17}, actively monitor cognate tRNA¹⁸, and that recognition of the correct codon–anticodon duplex induces an overall ribosome conformational change (domain closure)¹⁹. Here we propose an integrated mechanism for decoding based on six X-ray structures of the 70S ribosome determined at 3.1–3.4 Å resolution, modelling cognate or near-cognate states of the decoding centre at the proofreading step. We show that the 30S subunit undergoes an identical domain closure upon binding of either cognate or near-cognate tRNA. This conformational change of the 30S subunit forms a decoding centre that constrains the mRNA in such a way that the first two nucleotides of the A codon are limited to form Watson–Crick base pairs. When U•G and G•U mismatches, generally considered to form wobble base pairs, are at the first or second codon–anticodon position, the decoding centre forces this pair to adopt the geometry close to that of a canonical C•G pair. This by itself, or with distortions in the codon–anticodon mini-helix and the anticodon loop, causes the near-cognate tRNA to dissociate from the ribosome.

We determined six X-ray structures of the 70S ribosome at 3.1–3.4 Å resolution (Supplementary Tables 1 and 2) programmed by 30-nucleotide-long mRNAs with the AUG codon and tRNA^{Met} in the peptidyl tRNA-binding site (P site) and the A site occupied by tRNA^{Leu} or tRNA^{Tyr} (Fig. 1a and Methods). In one set of experiments, tRNA^{Leu} and tRNA^{Tyr} were bound to their respective cognate codons CUC and UAC in the A site. In a second set of experiments, we modelled near-cognate states of the ribosome (Supplementary Fig. 1). These states of the ribosome naturally occur during protein synthesis but with low probability because binding of cognate tRNA is kinetically

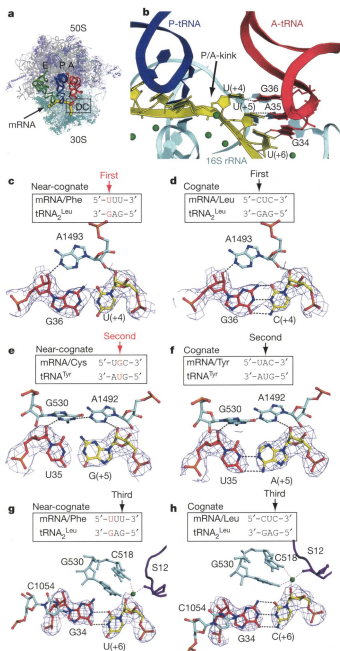


Figure 1 | Codon–anticodon interactions in the decoding centre on the 70S ribosome. **a**, The mRNA path on the 70S ribosome with the decoding (DC) area indicated. **b**, Close-up view of the mRNA P/A kink with near-cognate tRNA^{Leu}. Magnesium ions are in green. **c**, **d**, The first base pairs of the near-cognate (**c**) and cognate (**d**) codon–anticodon duplexes and their interactions with A1493 of 16S rRNA. **e**, **f**, The second base pairs of the near-cognate (**e**) and cognate (**f**) codon–anticodon duplexes and their interactions with G530 and A1492 of 16S rRNA. **g**, **h**, A classical wobble U•G pair (**g**) versus canonical C•G interactions (**h**); a magnesium ion interacting with the base pair is coordinated by protein S12 and part of 16S rRNA. All graphical representations were prepared with PyMol. Probable hydrogen bonds within 3 Å distance are indicated by dashed lines; 2F_o – F_c electron density maps are contoured at 1.2 sigma.

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favoured. We made the ribosome accept near-cognate tRNA by giving it only one type of tRNA carrying a mismatch to the A codon along with tRNA^{Met} for the P site. In these complexes the A site was filled either by tRNA^{Leu} and codon UUU with a U•G mismatch in the first position of the codon–anticodon helix or by tRNA^{Tyr} and codon UGC with a G•U mismatch in the second position.

As shown earlier, the mRNA forms a kink between the P and A codons (P/A kink), a universal feature of the mRNA path on the 70S ribosome that is stabilized by the P site tRNA, 16S ribosomal RNA (rRNA) and magnesium ions^{20,21} (Fig. 1b and Supplementary Fig. 2). The single mismatch states described above represent bona fide near-cognate complexes expected to have standard wobble U•G and G•U base pairs. At our data resolution (3.1–3.5 Å) we can confidently assign the general base pairing (Supplementary Figs 3 and 4). The electron density maps unambiguously demonstrate that U4 and G5 of the A codons UUU and UGC do not show the anticipated wobble interactions with G36 in tRNA^{Leu} and U35 in tRNA^{Tyr}, respectively. Instead, U4•G36 and G5•U35 at the first and second positions of

the codon–anticodon duplexes form base pairs similar to a standard Watson–Crick G•C pair (Fig. 1c, e and Supplementary Fig. 5). G•C-like G•U or G•T pairs have been shown earlier for RNA and DNA in other X-ray structures^{22,23}. When U•G is at the third codon–anticodon position we observe standard U•G wobble pairing (Fig. 1g, h). Unexpectedly, nucleotides A1493, A1492 and G530 of 16S rRNA in helix 44 (h44), which contact the first and the second pairs of the codon–anticodon helix, interact with these unusual U4•G36 and G5•U35 pairs identically to the way they interact with canonical Watson–Crick base pairs C4•G36 and A5•U35 (Fig. 1c–f). These findings are in contradiction with studies where these nucleotides were given a role as monitors and discriminators of canonical Watson–Crick pairs in the decoding process^{18,19}. Our structures show that G530, A1492 and A1493 form a static part of the decoding centre, defining its spatial and stereochemical properties (Fig. 2a, b).

The observed non-wobble U•G differs from previous X-ray studies that were based on the 30S subunit alone¹⁹ (Fig. 2c and Supplementary Figs 5 and 6). For example, for the study of the mismatch at the first

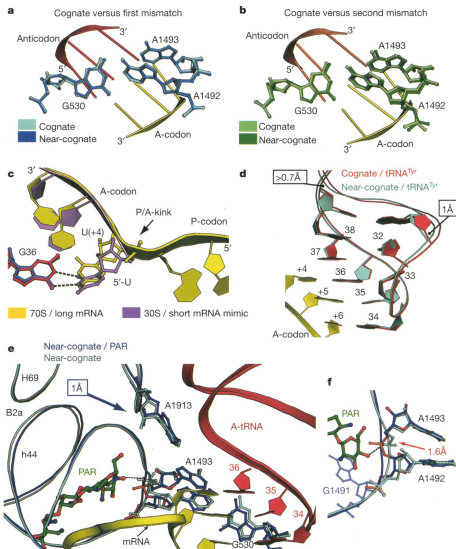


Figure 2 | The nature of the decoding centre. a, b, The overall conformations of universally conserved G530, A1492 and A1493 of 16S rRNA in the cognate structures are identical to those in the near-cognate models when the mismatches are at the first (a) or second (b) codon–anticodon positions. c, Differences between the position of the first uridine in the UUU codon base-paired to the GAG anticodon of tRNA^{Leu} from our 70S structure and from the 30S model¹⁹. d, Comparison of the anticodon loops of tRNA^{Tyr} in the cognate

(red) and near-cognate (cyan) states. e, Rearrangements of rRNA helices h44 and h69 in the near-cognate state upon binding of the aminoglycoside paromomycin (PAR). The near-cognate structures with tRNA^{Tyr} are shown (a similar effect of PAR is observed with tRNA^{Leu}, see also Supplementary Fig. 10). f, Magnified view of the changes in the A1493 phosphate position induced by PAR. Superimpositions in d, e and f were performed using 23S rRNA as reference.

position, 30S crystals soaked with an anticodon stem-loop of tRNA^{Leu} and a hexauridine (U₆) mRNA displayed a classical wobble U•G pair. There the P codon was mimicked by the 3'-end of 16S rRNA, so the U₆ mRNA could only bind to the A site and downstream, leading to a situation where the mRNA was not covalently linked between the P and A codons. By superimposing the A site GAG anticodons from our near-cognate tRNA^{Leu} structure and the 30S model we found that the first nucleotide of the A codon is positioned differently (Fig. 2c and Supplementary Fig. 6). Because it does not have the natural restraint coming from being covalently bound to the P codon, this first nucleotide in the A codon has the freedom to move so it can form a wobble U•G pair. However, in our structure the P/A mRNA link specifically directs the first nucleotide of the A codon to form Watson–Crick like interactions with G36 of tRNA^{Leu} (Fig. 1c).

The positional restrictions imposed by the decoding centre on the first two near-cognate U•G codon–anticodon pairs may result in differences in geometry compared with the corresponding cognate helices (Supplementary Figs 7 and 8). Although, the resolution of the data sets does not allow us to determine the exact value for these deviations in the base pairs, the tendencies are clear. A noticeable change is an increase in buckling at the first and third codon–anticodon positions with the U4•G36 mismatch (Supplementary Fig. 7) and at the second and third positions with the G5•U35 mismatch (Supplementary Fig. 8). These deviations could disturb the base stacking network within the near-cognate codon–anticodon helices and deform the entire anticodon loop structure²⁴ (Fig. 2d, Supplementary Fig. 9 and Supplementary Movie 1). This deformation might influence the position of helix 69 (H69) of 23S rRNA, whose universally conserved A1913 protrudes into the decoding centre^{20,21} (Fig. 2e).

Additional structures of the near-cognate states determined in the presence of the miscoding aminoglycoside paromomycin (Supplementary Tables 1 and 2) reveal a movement of H69 accompanied by

rearrangements of the intersubunit bridge B2a between h44 and H69 (Fig. 2e and Supplementary Movie 2). These distortions are most probably caused by binding of the antibiotic that strongly shifts the A1493 phosphate group (Fig. 2f). Although this shift does not alter much the interactions of A1493 with U4•G36 and A1492/G530 with G5•U35 (Supplementary Figs 7g and 8h), these local changes modulate the B2a bridge. H69 is displaced towards the tRNA, which probably enhances the interaction surface of H69 with the tRNA D-stem. In the presence of paromomycin the position of H69 is closer to that observed for the cognate state (Supplementary Fig. 10). Furthermore, displacement of the A1493 phosphate group relaxes the decoding pocket from the side of the A codon (Fig. 2e) and changes the deformation of the near-cognate codon–anticodon helix (Supplementary Movie 2). This novel understanding of the paromomycin action therefore differs from the previously suggested mechanism in decoding where it was proposed to influence the monitoring capabilities of A1492 and A1493 (ref. 18). The observed moderate structural rearrangements with paromomycin are consistent with its measured effect at the proofreading step²⁵.

We find that both near-cognate tRNAs induce rearrangements of the 30S subunit known as domain closure¹⁹ (that is, shoulder movement and head rotation) as described for cognate tRNA²¹ (Fig. 3a). This implies that domain closure is an inherent quality of the ribosome in response to binding of any tRNA to the A site¹² and is prerequisite for formation of the decoding pocket.

Initially, the mechanism underlying the decoding process was deduced from pioneer X-ray structures of the isolated 30S subunit where crystals were soaked with U₆ and anticodon stem-loop mimicking mRNA and tRNA^{18,19}. Besides those limitations, all attempts to model near-cognate states on the 30S subunit were performed in the presence of paromomycin, which, on the one hand, stimulated an ordered binding of the near-cognate tRNA analogs, but, on the other,

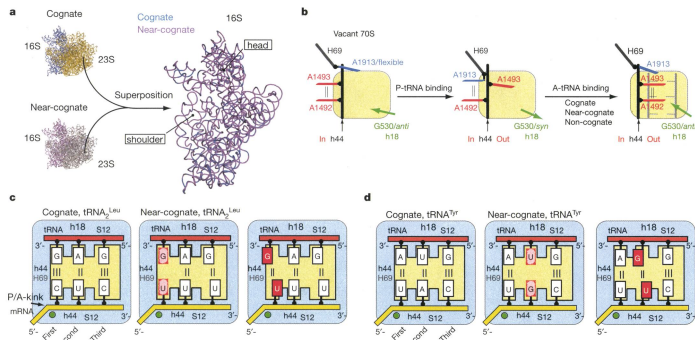


Figure 3 | The principle of decoding. **a**, Superposition of 23S rRNA from near-cognate and cognate structures with tRNA^{Leu} shows identical domain closure in the near-cognate and cognate states. **b**, Representation of conformations of the core nucleotides composing the decoding centre depending on its functional states (data with the vacant 70S ribosome are unpublished). **c**, **d**, Illustration of the decoding principle: together with the constraints imposed on the A codon by the P/A link coordinated by a magnesium ion (green sphere), the DC (h18, h44 and protein S12 from the

small subunit and H69 from the large subunit) restricts the allowed geometry of the first two nucleotides of the codon. No such restraints are imposed on the third base pair. A near-cognate tRNA with G•U in the first or second position is forced to form Watson–Crick-like base pairs (middle panels). This creates repulsion or requires energy for tautomerization (shown in pink), which by itself can be the source of the tRNA discrimination. The right panels illustrate the impossible situation when standard wobble base pairs (shown in red) occur in either the first or second positions of the codon–anticodon duplexes.

made it difficult to distinguish the independent effect of the mismatch on the 30S decoding centre¹⁹. The cognate and near-cognate models of the 70S ribosome described here, with or without paromomycin, together with our previous structures^{1,21}, give rise to novel insights into decoding with revisited roles for universally conserved nucleotides G530, A1492 and A1493 of 16S rRNA (Fig. 3b). Although these nucleotides form extensive contacts to the A-minor groove of a codon-anticodon helix, G530, A1492 and A1493 do not actively sense the correct Watson-Crick base-pairing geometry and thus do not discriminate against near-cognate tRNA.

We propose that upon binding of cognate or near-cognate tRNA to the 70S ribosome, the small subunit undergoes domain closure around the anticodon loop of the tRNA. The closure results in formation of a tight decoding centre that restricts the first two nucleotides of the A codon to form exclusively Watson-Crick base pairs with the tRNA anticodon (Fig. 3c, d). Owing to our current data resolution, we cannot precisely identify the hydrogen bond pattern of the mismatches in the near-cognate codon-anticodon helices, but tautomerism is a plausible chemical mechanism. An alternative explanation for the tRNA discrimination source could be repulsion in the U•G pair (Fig. 1c, e). Energy expenditure for formation of tautomers (or repulsion energy) could constitute the sole cause for the very efficient rejection of near-cognate tRNAs by the ribosome^{1,4}. Additionally, the observed deformation of the anticodon may lead to alterations in the codon-anticodon mini-helix and propagate through the rest of the near-cognate tRNA molecule, destabilizing it and causing the tRNA to dissociate from the ribosome (Supplementary Fig. 11). This corroborates the idea that evolutionarily tuned sequences of tRNAs play an active role in the tRNA selection process^{26–28}. Recent X-ray structures of the 70S ribosome with cognate tRNA and elongation factor Tu²⁸ demonstrated that binding of an anticodon loop of tRNA in the decoding centre is nearly identical to that shown for accommodated tRNA. This information prompts us to hypothesize that the same mechanism described here for the proofreading step governs the initial tRNA selection step.

METHODS SUMMARY

Ribosomes were purified from *Thermus thermophilus* cells as described before²¹. For all complexes, mRNA, tRNA^{Met} and tRNA^{Leu} (or tRNA^{Tyr}) were present in fivefold stoichiometric excess of the ribosome concentration. Complexes with paromomycin were obtained by including the antibiotic (30 μM) into the incubation mixture. Crystals were grown at 24 °C by sitting-drop vapour diffusion as described before²⁰. All crystals belonged to space group *P*₂₁₂₁₂ and contained two molecules per asymmetric unit. A very low dose mode with high redundancy was used for data collection²⁹. The structure¹, with tRNAs, mRNA and metal ions removed, was used for refinement with Phenix³⁰. Throughout refinement, no base-pair restraints were used between tRNAs and mRNAs to avoid bias towards standard base-pair geometry.

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Author Contributions N.D. and L.J. conducted experiments and performed analysis. All authors discussed the results and commented on the manuscript.

Author Information The atomic coordinates and structure factors for the determined crystal structures are deposited in the Protein Data Bank under accession numbers 3TVF and 3TVE (cognate tRNA^{Met} complex), 3UYE and 3UYD (near-cognate tRNA^{Leu} complex), 3UZJ and 3UZL (near-cognate tRNA^{Leu} complex with paromomycin), 3UZ6 and 3UZ9 (cognate tRNA^{Tyr} complex), 3UZG and 3UZF (near-cognate tRNA^{Tyr} complex), and 3UZL and 3UZK (near-cognate tRNA^{Tyr} complex with paromomycin). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to M.Y. (marat@igbmc.fr) or G.Y. (gula@igbmc.fr).

Telomerase RNA biogenesis involves sequential binding by Sm and Lsm complexes

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In most eukaryotes, the progressive loss of chromosome-terminal DNA sequences is counteracted by the enzyme telomerase, a reverse transcriptase that uses part of an RNA subunit as template to synthesize telomeric repeats. Many cancer cells express high telomerase activity, and mutations in telomerase subunits are associated with degenerative syndromes including dyskeratosis congenita and aplastic anaemia. The therapeutic value of altering telomerase activity thus provides ample impetus to study the biogenesis and regulation of this enzyme in human cells and model systems. We have previously identified a precursor of the fission yeast telomerase RNA subunit (TER1)¹ and demonstrated that the mature 3'-end is generated by the spliceosome in a single cleavage reaction akin to the first step of splicing². Directly upstream and partly overlapping with the spliceosomal cleavage site is a putative binding site for Sm proteins. Sm and like-Sm (Lsm) proteins belong to an ancient family of RNA-binding proteins represented in all three domains of life³. Members of this family form ring complexes on specific sets of target RNAs and have critical roles in their biogenesis, function and turnover. Here we demonstrate that the canonical Sm ring and the Lsm2-8 complex sequentially associate with fission yeast TER1. The Sm ring binds to the TER1 precursor, stimulates spliceosomal cleavage and promotes the hypermethylation of the 5'-cap by Tgs1. Sm proteins are then replaced by the Lsm2-8 complex, which promotes the association with the catalytic subunit and protects the mature 3'-end of TER1 from exonucleolytic degradation. Our findings define the sequence of events that occur during telomerase biogenesis and characterize roles for Sm and Lsm complexes as well as for the methylase Tgs1.

In eukaryotes, seven Sm proteins (SmB, SmD1, 2 and 3, SmE, SmF and SmG) form a heteroheptameric complex at U-rich Sm-binding sites (AU₄₋₆GR) of various small nuclear RNAs (snRNAs) including the spliceosomal snRNAs U1, U2, U4 and U5 (refs 4, 5). Assembly of Sm proteins *in vivo* requires the help of the survival of motor neuron protein (SMN), mutations in which result in spinal muscular atrophy⁶. At least two Sm-like complexes have been characterized. The Lsm1-7 complex functions in messenger RNA (mRNA) degradation^{7,8} and the Lsm2-8 complex is involved in the maturation of various polymerase III transcripts⁹⁻¹¹ and ribosomal RNAs¹². Purified Lsm2-8 binds to the 3'-terminal U-tract on U6, but not to the internal U-rich sites in U1, U2, U4 and U5 snRNAs, illustrating that Sm and Lsm complexes have different sets of target RNAs⁹.

Sm-binding sites are also found near the 3'-ends of telomerase RNA subunits from diverse yeasts^{12,13-16} and are important for RNA processing and/or stability^{12,13}. Actual binding of Sm proteins has been demonstrated for TLC1, the telomerase RNA from *Saccharomyces cerevisiae*¹⁵, but the functional consequences of this interaction have remained largely unexplored. The Sm-binding site in TLC1 is located several nucleotides upstream of the mature 3'-end¹⁵. In contrast, spliceosomal cleavage of *Schizosaccharomyces pombe* TER1 truncates the putative Sm-binding site by one nucleotide², which may compromise stable association of the Sm ring with mature TER1. We therefore set

out to examine which proteins bind to the 3'-end of mature TER1, and to determine the function of the putative Sm site for TER1 biogenesis and stability.

A strategy was developed to examine the 3'-end of TER1 by massively parallel sequencing to obtain a quantitative measure of 3'-end sequence distribution and to identify the most abundant terminal sequences (Fig. 1a). This analysis revealed that, after spliceosomal cleavage, over 60% of TER1 molecules lost additional nucleotides at the 3'-end and terminate in a stretch of three to six uridines (Fig. 1b). The 3'-end of most of TER1 therefore resembles the 3'-end of U6 snRNA, which is bound by the Lsm2-8 complex. To test whether Sm or Lsm proteins associate with TER1, carboxy-terminal c-Myc epitope tags were inserted at the genomic loci of all Sm and Lsm proteins.

Immunoprecipitations were performed with a subset of strains that did not show overt growth defects, expressed tagged proteins and maintained telomeres (Supplementary Fig. 1). The snRNA U1 control specifically co-precipitated with Sm proteins, confirming that the epitope tags did not interfere with immunoprecipitation of RNP complexes (Fig. 1c). TER1 co-immunoprecipitated with all four Sm

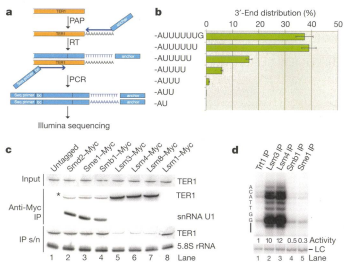


Figure 1 | TER1 RNA associates with Sm and Lsm proteins. **a**, Method used to map the 3'-end distribution of TER1 post spliceosomal cleavage. RNA is depicted in orange, DNA in blue. PAP, poly(A) polymerase; RT, reverse transcription; PCR, polymerase chain reaction; bc, barcode. **b**, Distribution of 3'-end positions in mature TER1 from wild-type cells. The average of four experiments is shown; error bars, standard deviation; a total of 23×10^6 sequences were scored. **c**, Northern blot of RNA isolated from immunoprecipitations with anti-c-Myc antibodies. Input and immunoprecipitation (IP) supernatant (s/n) represent 10% of the sample. An asterisk marks the position of the TER1 precursor. The lower band corresponds to the mature form of TER1. **d**, Telomerase activity assay performed on beads after c-Myc immunoprecipitation of tagged proteins as indicated above each lane. Activity was quantified relative to the Trt1 immunoprecipitate sample. A 100-mer [³²P]oligonucleotide was used as recovery and loading control (LC).

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proteins tested (Fig. 1c, lanes 2–4, and Supplementary Fig. 2a), including members of each of the three Sm subcomplexes¹. Strikingly though, several-fold more TER1 was recovered from Lsm immunoprecipitates resulting in an approximately 80% depletion of TER1 from the immunoprecipitation supernatant (Fig. 1c, lanes 5–7). TER1 precipitated with all subunits of the Lsm2–8 complex (Fig. 1c and Supplementary Fig. 2b), but not with Lsm1 (Fig. 1c, lane 8), the subunit specific to the Lsm1–7 complex.

To determine whether Sm and/or Lsm are associated with active telomerase, direct *in vitro* activity assays were performed on immunoprecipitates. Telomerase activity was detected in all samples, but was 20-fold higher in Lsm3 and 4 than Smb1 and Sme1 immunoprecipitates (Fig. 1d and Supplementary Fig. 2c). In part this can be explained by the lower recovery of telomerase with Sm proteins, as judged by quantification of telomerase RNA on northern blots (Supplementary Fig. 2c, d). However, even after normalization to the amount of TER1 in each immunoprecipitate, Lsm-associated telomerase activity was still 2.8-fold higher than that associated with Sm proteins. The simplest explanation for this observation is that a fraction of Sm-associated TER1 is not yet associated with the catalytic subunit of telomerase. Indeed, further experiments confirmed that Sm binding precedes Trt1 binding to TER1.

To gain insights into the functions of Sm and Lsm binding to telomerase we initially focused on the Sm association. For most characterized snRNAs, sequences downstream of the Sm-binding site are critical for Sm loading¹⁷. As the mature form of TER1 lacks such sequences, we tested whether the Sm complex was loaded onto the TER1 precursor before spliceosomal cleavage. Reverse transcription PCR (RT–PCR) confirmed that the precursor is indeed specifically associated with the Sm complex, but is undetectable in Lsm immunoprecipitates (Fig. 2a).

As the spliceosome contains Sm complexes, the TER1–Sm interaction may reflect binding of the spliceosome to the TER1 precursor. To test whether Sm proteins bind TER1 directly, we generated constructs with either a mutant 5′-splice-site or a deletion of the intron. Both mutant RNAs co-immunoprecipitated with Smb1 (Fig. 2b). In contrast, replacing the Sm-binding sequence with a random sequence (*ter1-sm6* mutant) reduced Sm association by 22-fold (Fig. 2c). Similarly, Lsm association was undetectable for *ter1-sm6* (Supplementary Fig. 3a). We therefore surmised that Sm and Lsm proteins directly bind to the previously identified site in TER1.

We next examined the effect of Sm binding on 3′-end processing by the spliceosome. Loss of Sm binding in the *ter1-sm6* mutant resulted in a sevenfold reduction in the processed form (Fig. 2d). Furthermore, a series of deletion mutants within the Sm site caused progressive inhibition of TER1 cleavage (Supplementary Fig. 3b), but not TER1 splicing (Supplementary Fig. 3c). Finally, introducing an eight-nucleotide spacer between the Sm site and 5′-cleavage-site also impaired processing (Fig. 2e). In summary, weakening or abolishing Sm association with the TER1 precursor reduces spliceosomal cleavage, indicating that Sm proteins promote 3′-end processing of TER1.

A conserved feature among yeast and mammalian telomerase RNAs is the post-transcriptional hypermethylation of the 5′-cap into a 2,2,7-trimethyl guanosine (TMG) form^{13,18}. Sm proteins were first implicated in promoting cap hypermethylation on U2 snRNA in *Xenopus* extract¹⁹. It was later shown *in vitro* that TMG-capping of human U1 requires the presence of SmbB/SmbD3 (refs 4, 20). A screen for physical association with Sm proteins led to the identification of the methylase Tgs1 in budding yeast²¹. To elucidate the roles of Sm and/or Lsm in the hypermethylation of the 5′-cap on TER1, we tested which, if any, of these proteins interact with *S. pombe* Tgs1 (ref. 22) by two-hybrid analysis. Smd proteins scored positive, with Smd2 displaying the strongest interaction, and the other Sm proteins and all Lsm proteins showing weak or no interaction (Supplementary Fig. 4a). We next examined whether preventing Sm binding to TER1 affects cap hypermethylation. Whereas wild-type TER1 was readily precipitated

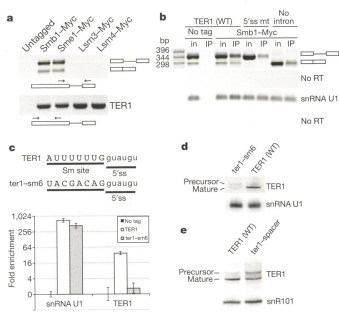


Figure 2 | Sm proteins associate with TER1 precursor and promote spliceosomal cleavage. **a**, RNA from anti-Myc immunoprecipitates was analysed by RT–PCR using primers in the first and second exon (primers represented by arrows in the schematic below the gel) to amplify the precursor form (upper panel). The primer pair also amplifies the spliced form (lower band in Sm immunoprecipitates). A primer pair in the first exon was used to visualize all forms of TER1 combined (lower panel). **b**, Sm association does not require spliceosome assembly on TER1. RT–PCR was performed on RNA purified from input (in) and anti-Myc immunoprecipitate beads (IP). Primers amplifying snRNA U1 were used as a positive control. **c**, The Sm-binding site (upper case) and 5′-splice-site (5′ss, lower case) for wild-type TER1 and the *ter1-sm6* mutant (MT). Replacing the Sm-binding site on TER1 (*ter1-sm6* mutant) compromises Sm association. RNA recovered from anti-Myc immunoprecipitates from untagged control and Smb1–Myc strains was quantified by real-time PCR. Data are plotted as enrichment over the untagged control. Error bars, standard error of triplicate experiments. **d**, Sm site mutation affects TER1 spliceosomal cleavage. Total RNA samples were analysed by northern blot for TER1 and snRNA U1. **e**, Increasing the distance between the Sm site and 5′-splice-site in the *ter1-spacer* mutant (AU₆GcgaucgUGU) impairs TER1 processing. Northern blot for TER1 and snoRNA snR101 as loading control.

with a monoclonal antibody against the TMG cap, *ter1-sm6* recovery was at least 25-fold reduced (Fig. 3a and Supplementary Fig. 4b). Only the cleaved form of TER1 was recovered in TMG immunoprecipitations from wild-type cells, suggesting that spliceosomal cleavage precedes hypermethylation (Supplementary Fig. 4c). TER1 was not TMG-capped in a *tgs1Δ* strain, confirming that Tgs1 is the enzyme responsible for TER1 cap hypermethylation (Supplementary Fig. 4d).

In light of the reported increase in telomerase RNA in *tgs1Δ* budding yeast²⁵, we were surprised to observe a fivefold reduction in mature TER1 RNA in *tgs1Δ* compared with wild type in *S. pombe* (Fig. 3b). In addition, an increase in the precursor indicated a 3′-end processing defect. The viability of *tgs1Δ* cells ruled out a major splicing defect, but we consistently noted a small reduction in spliceosomal snRNAs isolated from *tgs1Δ* cells (Fig. 3b and data not shown). To differentiate between a processing defect and a direct effect of the TMG cap on TER1 stability, we mutated the spliceosomal cleavage site and inserted a hammerhead ribozyme sequence to generate the mutant *ter1-5′ssmut-HH* (Supplementary Fig. 4e). In this construct, processing of TER1 occurs independently of the spliceosome by ribozyme cleavage. When comparing *ter1-5′ssmut-HH* levels between wild-type and *tgs1Δ* cells, a twofold reduction was observed (Fig. 3b). Taken together, these results show that *tgs1Δ* affects TER1 processing by the spliceosome as well as TER1 stability. Consistent with the exquisite dosage sensitivity for telomerase RNA in diverse species^{24,25}, this reduction in TER1

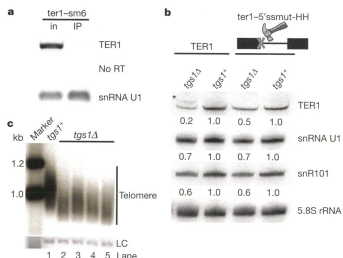


Figure 3 | Tgs1 modifies TER1 and is required for normal telomere maintenance. **a**, Loss of Sm site compromises TMG cap formation. RT-PCR amplifying all forms of TER1 and *ter1-sm6* mutant from anti-TMG immunoprecipitation (IP) and input (in) samples; snRNA U1 served as control. **b**, Bypass of spliceosomal cleavage reveals functions of Tgs1 in TER1 processing and stability. Northern blot analysis of TER1, snRNA U1, snR101 and 5.8S rRNA from total RNA prepared from wild-type and *tgs1Δ* strains harbouring either TER1 or the *ter1-5' smut-HH* mutant. An asterisk marks the position of the TER1 precursor. **c**, Deletion of *tgs1* causes telomere shortening. Telomere length was analysed by Southern blotting of EcoRI-digested genomic DNA from four independent *tgs1Δ* isolates and an otherwise isogenic *tgs1+* strain. A probe for the *rad16* gene was used as a loading control (LC).

resulted in shorter telomeres (Fig. 3c). Neither telomerase activity nor Lsm association was reduced beyond the effects expected from the reduced steady-state level of TER1 (Supplementary Fig. 4f, g).

Most TER1 post-spliceosomal cleavage was bound by Lsm2–8, but a small fraction was associated with Sm proteins (Fig. 1c). To investigate whether this was indicative of a switch from Sm to Lsm binding, we examined the distribution of 3'-ends in each immunoprecipitation by massively parallel sequencing. Around 70% of Sm-bound TER1 post-cleavage terminated precisely at the spliceosomal cleavage site (Fig. 4a and Supplementary Fig. 5a). Enrichment of this form in the Sm-bound fraction is consistent with Sm proteins binding the TER1 precursor and remaining associated with TER1 until after cleavage and cap hypermethylation have occurred. In contrast, Lsm-associated TER1 predominantly terminated in U_{3–6}, indicating that a switch between Sm and Lsm binding occurs after spliceosomal cleavage and is associated with exonucleolytic processing (Fig. 4a and Supplementary Fig. 5b).

Consistent with most telomerase activity being associated with Lsm2–8, the TER1 3'-end distribution from Trt1 immunoprecipitates was indistinguishable from that of Lsm-bound TER1.

The observation that loss of Sm binding coincided with the loss of terminal nucleotides led us to speculate that Lsm2–8 may function in protecting the 3'-end of TER1 against further exonucleolytic degradation. To test this, we attempted to generate Lsm deletion strains. Whereas most Lsm proteins are essential, *lsm1Δ* and *lsm3Δ* cells were viable. Consistent with a protective function for Lsm2–8, the levels of TER1 and U6 snRNA were reduced approximately fivefold in *lsm3Δ* cells (Fig. 4b). No such effect was seen when deleting *lsm1*, nor was the level of U1 snRNA reduced in *lsm3Δ* cells. The 3'-end sequence distribution for TER1 from total RNA of *lsm3Δ* cells closely resembled the Sm-bound fraction in wild type, whereas the Lsm-bound fraction was selectively lost in the mutant (Fig. 4c and Supplementary Fig. 5c). The viability of *lsm3Δ* cells further allowed us to confirm that cap hypermethylation is unaffected by the absence of Lsm consistent with Tgs1 acting on TER1 before Lsm binding (Supplementary Fig. 5d).

To verify independently a role for Lsm proteins in stabilizing TER1, we took advantage of the observation that Lsm binding requires a stretch of consecutive uridines⁹. In contrast, Sm binding tolerates other nucleotides in certain positions of the binding motif, as exemplified by the Sm-binding site in human U1 snRNA (AAUUGUG). When the TER1 Sm site was mutated to reduce the number of consecutive uridines, the level of mature TER1 was decreased (Fig. 5a). We next precipitated Smb1, Lsm4 and Trt1 from wild type and strains containing the *ter1-SmU1* mutant. As expected, the mutation had little effect on the binding of Sm proteins (Fig. 5b). In fact, when normalized for the lower level of *ter1-SmU1* compared with wild type, recovery of *ter1-SmU1* with Smb1 was increased 1.6-fold. In contrast, Lsm binding was diminished by more than 20-fold. Most surprisingly, the interaction between the catalytic subunit Trt1 and telomerase RNA was also compromised in the *ter1-SmU1* mutant (Fig. 5b). The normalized recovery of *ter1-SmU1* with Trt1 was 15-fold lower than wild type, indicating that Lsm binding facilitates Trt1–TER1 association, possibly by inducing a conformational change in the RNA analogous to how binding of the p65 protein facilitates telomerase assembly in *Tetrahymena*^{26–28}. Consistent with the poor recovery of *ter1-SmU1* in Trt1 immunoprecipitates, *in vitro* telomerase activity was below the level of detection (Fig. 5c).

Analysis of the 3'-end sequence distribution for *ter1-SmU1* from total RNA revealed that most of the mutant RNA ends at the cleavage site (Supplementary Fig. 6). This form constituted close to 90% of *ter1-SmU1* in Smb1 immunoprecipitates. In contrast, Lsm4 and Trt1 immunoprecipitates predominantly recovered RNA ending in -AUUU and -AUUGU (Supplementary Fig. 6). These results further support that Trt1 preferentially associates with Lsm-bound telomerase RNA.

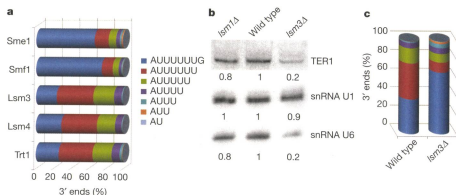


Figure 4 | Lsm proteins replace Sm and protect the 3'-end of TER1. **a**, 3'-End sequence distribution of TER1 from immunoprecipitation samples. **b**, Northern blot analysis from total RNA prepared from wild-type, *lsm1Δ* and *lsm3Δ* strains, quantified relative to wild type for each RNA. **c**, Specific loss of

Lsm2–8-bound fraction of TER1 in *lsm3Δ* cells based on 3'-end sequence analysis from total RNA samples. The wild-type sample from Fig. 1b is included for comparison.

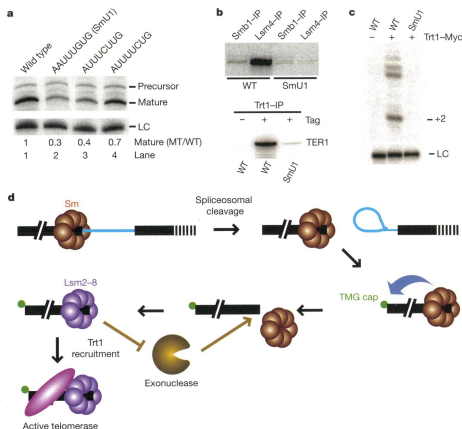


Figure 5 | Lsm binding to TER1 promotes telomerase assembly and protects TER1 from degradation. **a**, Northern blot for TER1. The indicated ratios of mutant (MT) to wild type (WT) are normalized to the loading control snR101 (LC). **b**, Northern blot for TER1 and the *ter1*-SmU1 mutant using RNA isolated from anti-c-Myc immunoprecipitations performed on extract from

They also confirm the role of Lsm in protecting the 3'-end of TER1 from further degradation, as diminished Lsm binding coincides with an overall reduction in telomerase RNA and a shift towards the form that is bound by Sm.

Taken together, our observations demonstrate that distinct populations of TER1 molecules associate with the Sm and Lsm complexes and suggest a sequence of events for TER1 biogenesis (Fig. 5d). The polyadenylated TER1 precursor is bound by the Sm complex, which promotes spliceosomal cleavage and subsequent 5'-cap hypermethylation by recruiting Tgs1. The Sm ring is then replaced by the Lsm2-8 complex, which protects TER1 from exonucleolytic degradation and promotes binding of the catalytic subunit.

Despite their structural similarity and related binding motifs, Sm and Lsm complexes have different modes of RNA binding and were thought to have distinct and non-overlapping sets of target RNAs. The finding that the TER1 precursor is exclusively associated with the Sm complex, whereas most mature TER1 is bound by Lsm2-8, revealed that biogenesis of telomerase RNA involves both Sm and Lsm complexes. Considering the central roles that Sm and Lsm proteins play in RNA metabolism, it will be important to determine whether biogenesis of other non-coding RNAs also involves Sm- and Lsm2-8-bound stages. Furthermore, it is interesting to note that several human Sm/Lsm proteins have been reported to co-purify with telomerase^{29,30}, raising the possibility that these proteins also function in TMG cap formation and telomerase assembly in metazoans.

METHODS SUMMARY

Myc epitope tags were integrated at the genomic loci and immunoprecipitations were performed in whole-cell extracts with anti-c-Myc antibodies. The different forms of telomerase RNA were detected by northern blotting and RT-PCR. The

strains harbouring Smb1-Myc, Lsm4-Myc or Trt1-Myc as indicated.

c, Telomerase activity assay performed on Trt1 immunoprecipitates from strains harbouring either wild type or *ter1*-SmU1. An untagged Trt1 strain was used as negative control. **d**, Sequence of events that occur during telomerase biogenesis.

distribution of 3'-ends was assessed at single nucleotide resolution by preparing libraries of oligo(A)-tailed telomerase RNA and massively parallel sequencing.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Visualizing molecular juggling within a B₁₂-dependent methyltransferase complex

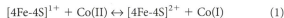
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Derivatives of vitamin B₁₂ are used in methyl group transfer in biological processes as diverse as methionine synthesis in humans and CO₂ fixation in acetogenic bacteria^{1–3}. This seemingly straightforward reaction requires large, multimodular enzyme complexes that adopt multiple conformations to alternately activate, protect and perform catalysis on the reactive B₁₂ cofactor. Crystal structures determined thus far have provided structural information for only fragments of these complexes^{4–12}, inspiring speculation about the overall protein assembly and conformational movements inherent to activity. Here we present X-ray crystal structures of a complete 220 kDa complex that contains all enzymes responsible for B₁₂-dependent methyl transfer, namely the corrinoid iron-sulphur protein and its methyltransferase from the model acetogen *Morella thermoacetica*. These structures provide the first three-dimensional depiction of all protein modules required for the activation, protection and catalytic steps of B₁₂-dependent methyl transfer. In addition, the structures capture B₁₂ at multiple locations between its 'resting' and catalytic positions, allowing visualization of the dramatic protein rearrangements that enable methyl transfer and identification of the trajectory for B₁₂ movement within the large enzyme scaffold. The structures are also presented alongside *in crystallo* spectroscopic data, which confirm enzymatic activity within crystals and demonstrate the largest known conformational movements of proteins in a crystalline state. Taken together, this work provides a model for the molecular juggling that accompanies turnover and helps explain why such an elaborate protein framework is required for such a simple, yet biologically essential reaction.

B₁₂-dependent methyl transfer lies at the heart of methylation biochemistry and is an essential reaction in human health and microbial CO₂ sequestration^{2,3}. In humans, methionine synthase (MetH) methylates homocysteine to form methionine to maintain cellular pools of folate (vitamin B₉) and S-adenosylmethionine (AdoMet), the universal methyl donor. MetH mutation or vitamin B₁₂ deficiency can cause serious health consequences, including megaloblastic anaemia and birth abnormalities such as neural tube defects¹³. Acetogenic bacteria, including *M. thermoacetica*, use the corrinoid iron-sulphur protein (CFeSP) and its methyltransferase (MeTr) together to catalyse methyl transfer in the Wood–Ljungdahl carbon fixation pathway for growth on CO₂ as the sole carbon source¹⁴.

For both MetH and CFeSP/MeTr, methyltetrahydrofolate (CH₃-H₄folate) is the methyl donor, and a protein-bound B₁₂ derivative (cobalamin for MetH and 5'-methoxybenzimidazolyl cobamide for CFeSP) is the methyl carrier. In acetogenic bacteria, the CH₃-H₄folate methyl group is derived from enzymatic reduction of CO₂, whereas in humans, CH₃-H₄folate is the predominant circulating form of the vitamin. Although CH₃-H₄folate is the common methyl source, methyl removal from the N⁵ tertiary amine is chemically challenging because the product, tetrahydrofolate (H₄folate), is a poor leaving

group¹. Therefore, a particularly powerful nucleophile is required, and B₁₂ with cobalt in the +1 oxidation state, a Co(I) species dubbed a 'supernucleophile'¹⁵, is recruited. Such strong reactivity comes at a price: reducing the inactive Co(II) state to active Co(I) is thermodynamically challenging, as the Co(II/I) reduction potential is one of the lowest in nature, −504 mV in CFeSP and −526 mV in MetH^{16,17}. In CFeSP, an electron is first delivered from a partner protein to an Fe₄S₄ cluster harboured by an activation domain^{18,19}. The electron is then passed to Co(II) to yield Co(I) (equation (1)), which attacks CH₃-H₄folate to form CH₃-Co(III) (equation (2)). CFeSP then delivers the methyl group to the Ni₂Fe₄S₄ active site metallocluster (A-cluster) of acetyl-CoA synthase (ACS), where it becomes the methyl of acetyl-CoA, and B₁₂ returns to its nucleophilic Co(I) state.



During the catalytic cycle of both MetH and CFeSP/MeTr (Supplementary Fig. 1), a series of 'molecular juggling' acts must be performed in which domains rearrange to contact the B₁₂ cofactor. Crystal structures of a MetH B₁₂-binding fragment⁴ and CFeSP from *Carboxydotherrmus hydrogeniformans* (ChCFeSP)⁹ both depict a 'resting' state, where B₁₂ is buried by a protective 'capping' domain, shielded from unwanted chemistry but inaccessible to substrate. Because methyl transfer uses S_N2 substitution²⁰, large conformational changes must 'uncap' B₁₂ before chemistry can occur. B₁₂ is 'uncapped' in structures of MeTr fragments that depict B₁₂ activation^{7,11,12}, but no structure has been solved that shows B₁₂- and CH₃-H₄folate-binding domains together to illustrate methyl transfer.

To visualize this elusive methyl transfer complex, we determined a 2.38 Å resolution structure of folate-free CFeSP/MeTr from *M. thermoacetica* (Fig. 1 and Supplementary Table 1). The homodimeric MeTr component (58 kDa) is virtually identical to previous structures of both MeTr¹⁰ (Supplementary Fig. 2a), root mean squared deviation (r.m.s.d.) for Cα atoms 0.39 Å, and the analogous MetH domain that binds CH₃-H₄folate⁴, r.m.s.d. 1.03–1.08 Å. MeTr and MetH both use (β/α)₈ triosephosphate isomerase (TIM) barrels to bind and activate CH₃-H₄folate for nucleophilic attack. Two CFeSPs are present in the complex, each containing two subunits. The small subunit (35 kDa) is a TIM barrel which acts as the B₁₂ 'cap' in the ChCFeSP structure⁹, while the large subunit (48 kDa) has three domains joined by linkers: an amino (N)-terminal Fe₄S₄ activation domain (residues 1–57), a TIM barrel domain (residues 93–312) and a carboxy (C)-terminal B₁₂-binding domain (residues 325–446). With the exception of the Fe₄S₄ and B₁₂ domains, discussed below, both CFeSP copies align well to the ChCFeSP structure, r.m.s.d. 0.81–0.85 Å (Supplementary Fig. 2b).

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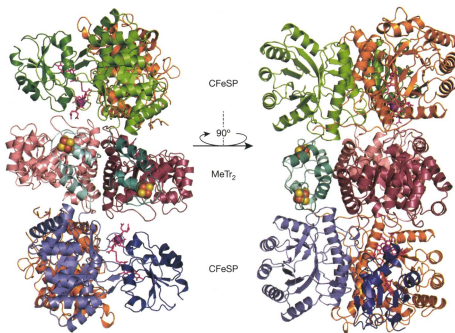


Figure 1 | The overall CFeSP/MeTr complex. Ribbon representation of MeTr homodimer (MeTr₂) in light and dark pink, CFeSP small subunits in orange, CFeSP large subunit Fe₄S₄ domains in teal and cyan, TIM barrel domains in

In the 220 kDa CFeSP/MeTr assembly (Fig. 1), the MeTr homodimer lies in the centre, with one CFeSP bound on either side. Each MeTr monomer has a C-terminal α -helix (residues 255–262) protruding from the TIM barrel rim. Contacts between this helix and its preceding loop with a CFeSP small subunit helix (residues 191–204) form the primary interactions between MeTr and CFeSP (Supplementary Fig. 3). Weak interactions between MeTr and CFeSP Fe₄S₄ domains have stabilized these highly flexible² domains responsible for B₁₂ activation, allowing their visualization as bundles of short α -helices connected by long loops that coordinate the Fe₄S₄ cubane (Fig. 1 and Supplementary Figs 4 and 5). The Fe₄S₄ domains are observed to adopt a variety of positions that are all too far from the B₁₂ to afford redox activation³. However, the long and primarily unstructured protein linkers that connect both the Fe₄S₄ and B₁₂ domains to the central TIM barrel must allow for the requisite flexibility for B₁₂ activation (Supplementary Figs 2b and 6).

B₁₂ domains of both MetH and CFeSP adopt Rossmann-like architectures that bind B₁₂ in the base-off conformation (Fig. 1)^{4,9}. High *B*-factors support the notion of flexibility mentioned above (Supplementary Fig. 7 and Supplementary Table 2), where electron density for both B₁₂ domains represents a highest occupancy position within an ensemble, rather than a sole conformation. In both CFeSPs, the average B₁₂ domain position resides between the ‘capping’ small subunit TIM barrel and the TIM barrel of a MeTr monomer, which are adjacent and nearly perpendicular to each other (Fig. 1). On average, the B₁₂ Co has shifted approximately 6.5 Å away from its ‘resting’ location towards the MeTr folate-binding site. B₁₂ in this structure is thus positioned ‘en route’ towards catalysis, with approximately 18 Å remaining to the methyl group of folate modelled into the MeTr active site, based on an alignment with the folate-bound MeTr structure¹⁰. In transitioning between ‘resting’ and ‘en route’ positions, the B₁₂ corrin ring breaks three interactions with the ‘capping’ domain and forms new contacts, including an H-bond with Asn 203 of MeTr (Fig. 2a, b).

Given the flexibility suggested by this structural analysis, we explored whether the B₁₂ domain can sample the 18 Å necessary to afford turnover within intact crystals, using anaerobic *in crystallo* ultraviolet–visible absorption spectroscopy to monitor the state of B₁₂. *In crystallo* and analogous solution spectra were collected in

green and blue, and B₁₂ domains in dark green and dark blue. B₁₂ cofactors in magenta sticks with cobalt as violet spheres. Fe₄S₄ clusters in spheres: Fe in orange, S in yellow.

parallel (Fig. 3) for the as-isolated Co(II) form of B₁₂. Reduction to Co(I) and methylation to CH₃-Co(III) were then achieved *in crystallo* and in solution, with all spectra matching well-established CFeSP absorption features^{18,19,21–23}. Importantly, these features disappear when light is passed through the solution surrounding the crystals, indicating that spectra represent protein in crystals and not protein that may have been liberated into the solution. Collectively, these data demonstrate enzymatic transfer of the CH₃-H₄folate methyl group to CFeSP-bound B₁₂, evidence that the B₁₂ domain is able to move at least 18 Å to trigger methyl transfer within the crystal. To our knowledge, this conformational movement represents the largest observed in a crystallized protein (Supplementary Discussion). Such dramatic B₁₂ domain movement is probably facilitated by the fact that CFeSP/MeTr is mostly composed of rigid TIM barrels that provide all the lattice contacts (Supplementary Fig. 8). Although their biosynthesis is energetically expensive, these high molecular mass TIM barrel scaffolds may be important for B₁₂-dependent methyltransferases to maintain structural integrity during the conformational gymnastics that alternately enable activation, protection and catalysis of the highly reactive B₁₂ cofactor. Thus, despite the small size of the transferred methyl moiety, these large conformational changes appear to necessitate large enzyme sizes.

Although the folate-free CFeSP/MeTr structure describes large B₁₂ domain movements that ‘uncap’ B₁₂ from the small subunit, it is interesting to consider why binding of CFeSP to MeTr does not simply position the B₁₂ domain directly over the MeTr active site. One explanation is that the structure represents an inactive complex; however, *in crystallo* results clearly demonstrate that CFeSP/MeTr crystals are active. Another explanation posits that an ensemble of ‘en route’ conformations exists when CH₃-H₄folate is absent, and that CH₃-H₄folate binding would shift the conformational equilibrium, moving B₁₂ closer to the folate-binding site. To obtain experimental support for this hypothesis, we solved additional CFeSP/MeTr structures co-crystallized with CH₃-H₄folate, with and without Ti(III) citrate as a reductant at 3.03 Å and 3.50 Å resolution, respectively. Absorption spectroscopy performed on these crystals shows that these structures represent a substrate form (CH₃-H₄folate bound, B₁₂ in the Co(II) state) and a product form (H₄folate bound, B₁₂ in the CH₃-Co(III) state) of the complex (Supplementary Fig. 9). Compared with

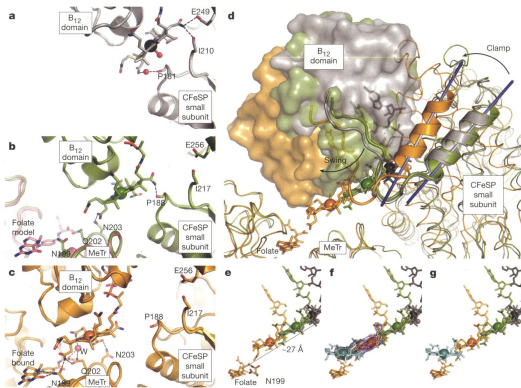


Figure 2 | Comparison of B_{12} positions in 'resting' ChCFeSP, folate-free and folate-bound CFeSP/MeTr. **a**, ChCFeSP (grey ribbons, Co of B_{12} : black sphere). **b**, Folate-free CFeSP/MeTr (green ribbons, Co of B_{12} : green sphere) superimposed with CH_3-H_4 folate-bound MeTr (Protein Data Bank accession number 2E7F, pink ribbons). **c**, Folate-bound CFeSP/MeTr (orange ribbons, Co of B_{12} : orange sphere). Parts **a–c** are identical in orientation; B_{12} sticks coloured as C, ribbon colour; O, red; N, blue. **d**, Superposition of ChCFeSP (grey), folate-free CFeSP/MeTr (green), and folate-bound CFeSP/MeTr

(orange) structures in **a–c**, highlighting one helix (thick ribbons) to show clamping motion (helix axes as straight blue lines) and B_{12} (sticks) with 12-residue linker (thick ribbons) to B_{12} domain (surface) to show swinging motion. **e**, Superposition of B_{12} and CH_3-H_4 folate in **d**, with Asn 199 shown for CFeSP/MeTr structures in sticks (C, ribbon colour; O, red; N, blue). **f**, Same as **e**, with $2F_o - F_c$ density in blue (1.0σ) and pink mesh (4.0σ), and $F_o - F_c$ density in green mesh (3.0σ) for folate-bound CFeSP/MeTr structure. Putative alternative B_{12} corrin: cyan. **g**, Superposition of B_{12} cofactors and CH_3-H_4 folate in **f**.

the folate-free structure, B_{12} in both folate-bound structures has indeed moved even closer to the MeTr folate-binding site (by an average of 7.7 Å) and exhibits new H-bonding features (Fig. 2c). In these folate-bound structures, the B_{12} corrin ring has severed all interactions with the 'capping' CFeSP small subunit and contacts only MeTr residues. Here, asparagine and glutamine residues that line the MeTr surface appear to participate in an 'amide hand off', sequentially passing B_{12} along its trajectory as it progresses towards folate (Fig. 2b–c and Supplementary Fig. 10).

Interestingly, the terminal amide in this 'hand off', Asn 199, is strictly conserved in both MeTr and MeTh and was previously shown to switch conformations between folate-free and folate-bound forms¹⁰, a feature also observed in the CFeSP/MeTr structures presented here (Fig. 2b, c, e). In apo-MeTr structures, Asn 199 points upwards and out of the active site, whereas in folate-bound MeTr structures Asn 199 turns down to H-bond with the N^5 of folate. Because N199A mutation moderately hinders folate binding (20-fold in dissociation constant, K_d) but dramatically compromises catalytic efficiency (k_{cat}/K_m) by 25,000-fold, Asn 199 is thought to be important for formation of the transition state¹⁰. In our CFeSP/MeTr structures, we observe a new role for Asn 199 in B_{12} domain conformational switching: when folate is absent, Asn 199 points out of the active site, blocking a closer B_{12} position. However, when folate binds and Asn 199 reorients to H-bond with folate, space becomes available for B_{12} to move closer to the MeTr folate-binding site. Therefore, the position of Asn 199 itself could help shift the conformational equilibrium of the B_{12} domain, signalling that substrate has bound to MeTr. Asn 199 is an ideal signal for substrate binding, as it is the only MeTr residue known to reposition upon folate binding¹⁰.

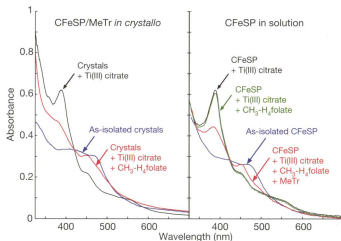


Figure 3 | Methyl transfer activity of CFeSP/MeTr crystals by ultraviolet-visible absorption spectroscopy. As-isolated spectra (blue lines) for CFeSP/MeTr crystals and CFeSP in solution similarly have broad features at approximately 400 and 470 nm arising from the Fe_S cluster and the B_{12} corrin. Following established protocols^{18,19,22,23,26,27}, B_{12} reduction was achieved with $Ti(III)$ citrate, yielding a sharp 390 nm peak indicative of active Co(I) in both solution and in *crystallo* spectra (black lines). Further treatment with CH_3-H_4 folate yields decreased absorbance at 390 nm and a new peak at 450 nm (red lines), characteristic of the product complex (protein-bound $CH_3-Co(III)^{18,(19,22,27)}$). A control reaction (green line) confirms that turnover does not occur from free CH_3-H_4 folate without MeTr, and the 450 nm peak indicates that B_{12} remains CFeSP-bound (free B_{12} has a peak at approximately 520 nm instead^{18,21,27}).

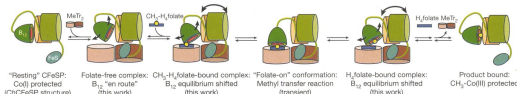


Figure 4 | Cartoon model of B_{12} -dependent methyl transfer in CFeSP/MeTr. For simplicity, only one of the two CFeSP heterodimers is shown. Protein domains are coloured as in Fig. 1, loops represent linkers, red hexagon

Displacement of the B_{12} domain from its resting position to the position nearest the folate-binding site can be attributed to two independent conformational changes within the complex, best described as 'swinging' and 'clamping' motions (Fig. 2d). The B_{12} domain can 'swing' relative to the rest of CFeSP (Supplementary Fig. 11), and CFeSP can 'clamp' the B_{12} domain towards the MeTr active site (Fig. 2d and Supplementary Fig. 12). Despite varying degrees of 'clamping' over a range of approximately 14° across the structures, the interface between the CFeSP small subunit and MeTr is preserved (Supplementary Fig. 13).

Although folate binding shifts the average position of the B_{12} domain closer to the MeTr folate-binding site, the B_{12} Co is still too far for S_N2 methyl transfer (Fig. 2e). Intriguingly, a large, continuous electron density peak is present in $2F_o - F_c$, $F_o - F_c$, and composite omit maps, emanating from the corrin ring and stretching directly over the folate-binding site, suggestive of an alternative, low-occupancy corrin conformation (Fig. 2f and Supplementary Fig. 14). A trial refinement of a putative corrin ring at 40% occupancy satisfies the $F_o - F_c$ difference maps (Supplementary Fig. 15), positioning B_{12} over the folate-binding site.

The multiple positions of B_{12} captured here (Fig. 2g) highlight the conformational flexibility of the CFeSP/MeTr scaffold and provide a framework to understand the molecular juggling of domains during B_{12} -dependent methyl transfer (Fig. 4). Before MeTr binding, the CFeSP B_{12} domain rests against the 'capping' small subunit, as in the structure of ChCFeSP, with reactive Co(I) of B_{12} protected ('resting' state). From this conformation, either the 'cap' or the B_{12} domain must move to allow substrate access. Our folate-free CFeSP/MeTr structure indicates that upon MeTr binding, the B_{12} domain becomes 'loosened' and flexible, adopting an ensemble of conformations that lie en route towards the MeTr active site. Here, the reactive B_{12} species would be protected by the CFeSP small subunit and MeTr TIM barrels. CH_3 - H_4 folate binding to MeTr accompanied by movement of Asn 199 shifts the equilibrium of B_{12} domain conformers, placing B_{12} closer to folate, as in our folate-bound CFeSP/MeTr structures, with B_{12} protection afforded by MeTr. It is notable that even after CH_3 - H_4 folate binds, the major B_{12} position is still not directly over the folate methyl group, as such a position is expected to be transient. After methyl transfer, the B_{12} domain can return to the small subunit to 're-cap' the methylated B_{12} product, protected by the small subunit TIM barrel.

Overall, our data indicate that B_{12} domain movement is not a simple, two-state switch between 'resting' and 'catalytic' conformations. Instead, a flexible B_{12} domain samples an ensemble of conformations, where subtle shifts of the conformational equilibrium place B_{12} progressively closer to the active site, thereby increasing the population of conformers capable of methyl transfer without obstructing substrate access or hindering domain movement. This model is consistent with MeTr studies where ligation, alkylation and redox state of the B_{12} cobalt can favour/disfavour various binding modes, alternately shifting the equilibrium of conformers for ordered domain rearrangements during the reaction cycle^{1,12,24,25}. We further identify MeTr residues that contact B_{12} along its trajectory, ending with Asn 199. In MeTr the B_{12} ligating residue His759 has been shown to play a dual role in catalysis and in signalling conformational shifts¹. The strictly conserved, folate-binding Asn 199 of MeTr could similarly play a dual role in both catalysis and conformational signalling. We thus expect this

is B_{12} , blue rectangle is folate and transferred methyl group is shown as a yellow sphere. Curved arrows denote 'swinging' and 'clamping' motions.

model for dynamic domain juggling, communicated by residues involved in substrate and cofactor binding, to be a common theme in methyl transfer between the B vitamins folate and B_{12} .

METHODS SUMMARY

CFeSP and MeTr were expressed and purified anaerobically from *M. thermoacetica* ATCC 39073 and from recombinant *Escherichia coli*, respectively. Crystals were grown anaerobically by hanging drop vapour diffusion. Diffraction data were collected at 2410°C at the Advanced Photon Source, Argonne National Laboratory, and 5.02 and 8.22 Å at the Advanced Light Source, Lawrence Berkeley National Laboratory. Structures were solved by molecular replacement. Data collection and refinement statistics are presented in Supplementary Table 1, and representative electron densities for protein domains and for cofactors/substrate are shown in Supplementary Figs 16–21. Solution and *in crystallo* absorption spectra were collected as described in the text and in the Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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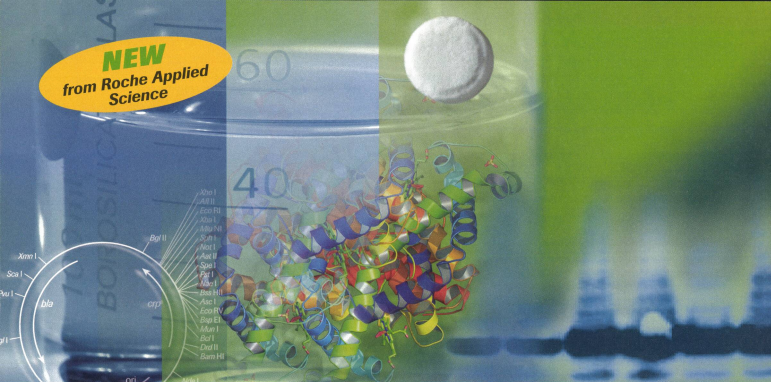
Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Atomic coordinates are deposited in the Protein Data Bank under accession codes 4DJJ, 4DJF and 4DJF. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to C.L.D. (cdrennan@mit.edu).

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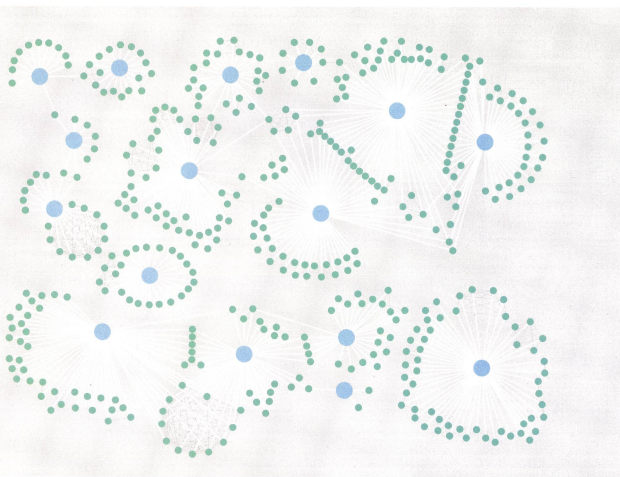
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THE INTERACTION MAP

As increasing numbers of protein–protein interactions are identified, researchers are finding ways to interrogate these data and understand the interactions in a relevant context.

M. SHALES/KROGAN LAB/UCSF



Multiple replicated experiments and sophisticated statistics reveal 497 interactions between 16 HIV proteins (blue) and hundreds of human factors.

BY MONYA BAKER

Around the time that scientists celebrated the completion of the draft sequence of the human genome, papers from two separate groups described results of another project that tested all the possible pairings of thousands of yeast proteins to see whether they interact^{1,2}.

The importance of protein–protein interactions is beyond dispute. Little happens in a cell without one protein ‘touching’ another. Whether a cell divides, secretes a hormone or triggers its own death, protein–protein interactions make the event happen. Consequently, comprehensive maps

showing which proteins came together in a yeast cell were much anticipated.

But the results took scientists aback. Although the two research groups had explored the full collection of proteins in the same organism using the same yeast two-hybrid (Y2H) assay, the two papers found fewer than 150 interactions in common — only 10% of the findings that either team dubbed high quality. Most scientists regarded the results as so riddled with artefacts that they were useless.

“As you can imagine, people were extremely critical. They just couldn’t believe that you would get such different results when you were studying the same thing,” recalls Peter

Uetz, who studies protein interactions at the Center for the Study of Biological Complexity at Virginia Commonwealth University in Richmond, and was a co-author on one of the papers¹. Even today, many researchers look askance at the Y2H assays used in the studies.

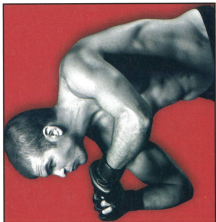
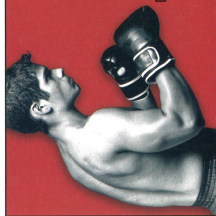
But Marc Vidal, a systems biologist at the Dana-Farber Cancer Institute in Boston, Massachusetts, says that the technique has come a long way in a decade. Not only have researchers found ways to recognize and reduce false-positives, but gruelling follow-up studies show that the startlingly low overlap between the two reports was not because the assays found so many interactions that do not exist, but because they missed so many that do³. ►

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► Understanding these interactions is as important as ever. Protein interactomes — maps of protein interactions — are raw fuel for systems biologists. Promising techniques to block protein–protein interactions in cancer cells and for other diseases have launched a string of biotechnology deals. Considering disease in terms of protein–protein interactions rather than individual genes and proteins could help to untangle jumbled observations. For example, mutations in the same protein could lead to different diseases by disrupting different interactions. Similarly, mutations in different proteins that disrupt the same interaction could lead to the same disease.

A good reference map of interactions would be like completing the human genome sequence, says Vidal, and could spawn further efforts to study genetic variation and function. A validated network would give scientists a jumping off point for more experiments. “My guess is that as these networks grow, we will get more elaborate ways of understanding where these interactions take place, when and why,” he says. “We are getting a sense of a cell’s organizational self by doing this.”

SCREENING SYSTEMS

First described in 1989, the Y2H assay tests the interactivity of pairs of proteins by attaching them to two halves of a transcription factor¹. If the proteins come together, the transcription factor is reformed, activating reporter genes and allowing the yeast to grow. Companies including Hybrigenics in Paris and Dualsystems Biotech in Zurich, Switzerland, run Y2H as a service.

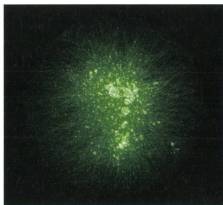
“Yeast two-hybrid has an enormous advantage, which might also be a disadvantage: it can detect low affinity,” says Erich Wanker, a neuroproteomics researcher at the Max Delbrück Center for Molecular Medicine in Berlin, and co-editor of a book on the topic². In other words, the assay can identify weak, transient pairings such as those that perpetuate cell signalling. But it also detects proteins that randomly bump together. This bumping has led to almost philosophical discussions. “At what point do we really believe that it’s an interaction?” asks Wanker.

Scientists have also found ways to detect and avoid many sorts of false positive. Artefacts from ‘sticky’ proteins, which bind non-specifically to other proteins, can be identified and excluded. Growth that is promoted by a single introduced protein rather than a reformed transcription factor can also be recognized.

Precise systems also exist to make sure that all desired combinations are tested. Rather than transfecting the same yeast cell with genes for two potential interaction partners, yeast are transacted with individual genes, mated in pools and their progeny assayed for growth. Robotic systems mix yeast precisely and run multiple replicates of each assay. The

number of times that the same interaction is seen becomes part of a quality score. “Our view is that Y2H can give reliable and reproducible results,” says Wanker.

Still, some interactions will not be observed in Y2H. For example, the interacting proteins have to allow the two halves of the transcription factor to reunite, and the proteins must be able to reach the nucleus to activate the reporter gene. Thus, interactions



Interaction maps can help to explain protein function and identify new ways to fight disease.

with membrane- or organelle-specific proteins are invisible.

Besides Y2H, lower-throughput tests in mammalian cells can be used to screen interactions; these tests include luminescence-based mammalian interactome (LUMIER), mammalian protein–protein interaction trap (MAPPIT), protein arrays and protein-fragment complementation assay (PCA). Although these are orders of magnitude slower than Y2H, they can probe interactions in a more relevant context.

MAPPIT is one of the highest-throughput mammalian screens. Instead of a yeast transcription factor, a mammalian cytokine receptor is split and becomes capable of cell signalling only when reconstituted. In 2009, Jan Tavernier, a network biologist at VIB, a life-sciences research institute in Ghent, Belgium, described a higher throughput version of MAPPIT in which plasmids encoding potential interaction partners linked to one cytokine receptor fragment can be individually spotted into wells and stored³. To begin the experiment, wells are filled with cells expressing the cytokine-receptor fragment linked with the selected ‘bait’. When interactions occur, signalling activates the light-emitting enzyme luciferase.

Using multiwell plates it costs about €2,000 (US\$2,600) to screen one bait protein against the human ORFeome (a complete set of cloned protein-encoding open reading frames), says Tavernier, who hopes to describe techniques to run MAPPIT on microchips later this year. Miniaturized assays should reduce the cost to €100 and allow the ORFeome to be tested

against 100 baits a week.

At this throughput and cost, Tavernier says, new kinds of experiments become feasible. Instead of restricting screens to yeast cells, “you start mapping full interactomes in the appropriate species”, he says. In addition, Tavernier plans to compare how interactomes change when cells are treated with agents such as drugs or toxic chemicals. He is hoping to commercialize the technology, and is working with Vidal and other scientists to map human protein interactions using both MAPPIT and Y2H assays.

LUMIER assays are also relatively high-throughput and can be used to test whether particular interactions are affected by drugs, hormones or other additives. For these assays, cells are transiently transfected with two proteins. One protein is attached to a hydrophilic peptide called FLAG. Potential interaction partners are linked with luciferase. Cells are lysed, the FLAG-tagged proteins are captured and the presence of the interacting partners can be detected by the light they give off⁴.

Protein-fragment complementation assays, which can be conducted in yeast as well as mammalian cells, rely on reconstituting a wide range of ‘reporters’, often enzymes or fluorescent proteins. Since the reporters can signal throughout the cell, interactions can be detected where they naturally occur.

In a collection of articles published in January 2009, Vidal, Wanker and others described what Vidal terms an empirical framework for assessing protein interactions found in high-throughput screens⁵. In practice, this means repeating experiments using different types of assay and comparing the results with sets of controls. The positive



“We are getting a sense of a cell’s organizational self by having a validation network”

Marc Vidal

controls are a reference set of about 100 well-established interactions carefully selected from the literature. The negative controls are some 100 randomly assigned pairs that have never been observed together. Conditions of the assays are adjusted to boost detection of positive controls without raising the detection of random interactions.

As part of a framework put forth in *Nature Methods*⁵, results from interaction studies should be confirmed in different types of assays. The more methods that find an interaction, the more confident researchers can be. Still, collectively, these assays detect only about 70% of the positive reference set (see ‘Beyond binary interactions’).



Yeast two-hybrid assays can probe hundreds of thousands of potential protein interaction pairs a week.

High-throughput experiments are not the only way to identify protein–protein interactions. Several databases, such as the Biological General Repository for Interaction Datasets (see thebiogrid.org) and IntAct (see www.ebi.ac.uk/intact), compile lists of interactions as they are published in the literature, culling from both small-scale

and high-throughput experiments as well as predicted interactions inferred from other analyses. But this list is not even close to complete, says Sandra Orchard, a proteomics service coordinator at the European Bioinformatics Institute in Hinxton, UK, who helped to develop minimal information standards to help share and evaluate interaction data. “We

will be lucky if as much as 30% of the yeast interactome has been observed,” she says. For the human interactome, she estimates that the figure is less than 10%, including published results that are not captured in the databases.

WHEN TO BELIEVE

Biologists rely on interaction data in several ways. They often layer protein–protein interaction networks onto other networks. After identifying transcription factors that regulate a gene, for example, they search databases and literature for transcription factors’ interaction partners. Researchers also explore how sets of proteins are connected to each other, and then ask questions based on the structure of the network, such as classifying the proteins that have the most interaction partners. But not all interaction data are equal, warns Russell Finley, a network biologist at Wayne State University School of Medicine in Detroit, Michigan, who believes that incorporating quality measures could make the data substantially more powerful. At present, he says, savvy researchers filter out interactions unless they have been observed more than once through different methods, but these ‘intuitive filters’ can be biased. For example, the more often a protein is studied,

Beyond binary interactions

Rather than individual proteins, protein complexes are the functioning biochemical entities in a cell, says Nevan Krogan, a systems biologist at the University of California, San Francisco. “If you think about a protein, more often than not it is in a protein complex.” If a complex requires multiple components to form, two-hybrid studies cannot be expected to detect it.

Instead, complexes are generally studied in ‘pull down’ assays. The gene for an interest is fused to a peptide tag that allows it to be ‘fished’ from cell lysates; less commonly, antibodies are used to purify out unlabelled proteins. The captured protein pulls its associates with it, and these are identified, usually by mass spectrometry. Companies such as Agilent Technologies in Santa Clara, California; Cell Signalling Technology in Danvers, Massachusetts; and IBA in Göttingen, Germany, offer transfection vectors and capture technologies.

Typically, researchers tag one protein of interest in each experiment, and these are then captured on a column. If not enough protein is captured, researchers can use experiments that add further tags and capture steps. If a complex is fragile, crosslinking reagents can be added to cells, binding nearby proteins together.

Although transient and fragile components

of complexes are hard to detect, the biggest problem in pull down assays is background — proteins that are not part of a complex but get pulled along. Researchers are getting much better at telling which proteins observed with a tagged protein are actually part of a complex, says Anne-Claude Gavin, who studies protein complexes at the European Molecular Biology Laboratory in Heidelberg, Germany.

The secret is statistics. If a protein complex has ten components, there should be ten ways to pull down the complex, explains Gavin. In a large data set involving many tagged proteins, she says, the same protein should occur in three forms: as the tagged protein, as an interaction partner and as background. Several scoring systems are used to sort artefacts from real interactions and to identify the components of protein complexes. The key, says Krogan, is to collect enough data. “For these scoring systems to be effective, you need pull downs of many types of proteins.”

Krogan and his colleagues¹² recently used pull down experiments to identify complexes formed between HIV proteins and host proteins. Disrupting these interactions could prevent the virus from entering cells. They selected 18 HIV proteins and tagged them in two ways, using FLAG and Strep tags. The

team developed a scoring system called mass spectrometry interaction statistics to sort the interactions. The system compiles a single score on the basis of abundance (more abundant proteins are more likely to be background), reproducibility and specificity. Almost 500 proteins reached this threshold; only 19 of these had already been reported¹².

This system vastly increased the number of potential targets for drugs that could treat HIV, and revealed a way to perform further studies on a previously intractable HIV protein — Vif, which was known to overcome human cells’ antiviral defences but was hard to study biochemically and structurally. The reason, says Krogan, was missing interaction partners. “You needed all the components of the complex there to make it behave.”

Now, researchers will be able to find potential ways to disrupt the complex, which could lead to new anti-HIV drugs. The real challenge, says Krogan, is to continue to mine the identified interaction partners for biological meaning: low-throughput work that requires extensive follow-up study. “The main goal of making these maps is not generating these maps. It is to extract biological insight, mechanism and testable hypotheses,” he says. “Sadly, work almost always stops at the maps.” **M.B.**

the more interactions will be found. Finley says that a better approach would be to consider all the data available and assign a score reflecting the likelihood that an interaction is real. Computer analyses could then be used to consider more interactions, giving more weight to those with higher confidence scores.

But an interaction can occur and have no actual consequences. "The real question is what interactions have meaning in the first place," says Stephen Michnick, a biochemist at the University of Montreal, Canada. "An interaction can be quite good, that is, reproducible in multiple assays, but not be biologically important." In other words, the interaction has no discernible effects: it does not start or stop a molecular machine, activate an enzyme or send another protein to destruction.

Michnick came to these conclusions after conducting a comprehensive study that allowed protein interactions to be studied in a more natural context. In the protein-fragment complementation assays, interacting proteins reconstituted an enzyme that yeast needed to survive under culture conditions⁸. This identified about 3,000 new interactions, with many involving membrane and other proteins that cannot reach the cell nucleus.

But thousands of other protein interactions were observed with less confidence. "We were surprised that there were known proteins that made too many interactions or made interactions that didn't make biological sense," Michnick recalls. "We thought we had the perfect method, and so we would get perfect results." "So we thought, if we are seeing junk interactions and other people are seeing junk, what is the junk?" The answer, he believes, is that these are naturally occurring 'junk' interactions that, like sections of DNA that do not seem to have a function, simply exist.

Michnick believes that perhaps as many as half of the interactions observed even in rigorous screens have no biological function. Abundant proteins should be treated with particular scepticism, but if the same pairs of proteins are consistently found together and not with other proteins then that interaction is more likely to be real, and the same is true of interactions identified across multiple species. "The parts that are functional have to be dissected from the rest of what's there," Michnick says.

Trey Ideker, a network biologist at the University of California, San Diego, is more worried that such a small percentage has been observed at all. "It's not clear how you can shortcut to the functional interactions without some unbiased way of getting all the interactions," he says. "We have a flashlight illuminating 20% of the yard, but the other 80% is dark." In fact, no one yet knows how big the universe of interactions is, he says, "but everyone agrees that we are not even close to having mapped it".

Nonetheless, more interactions have been

identified than can be individually investigated. For Ideker, the best approach is to think in terms of databases. "I have this big 'gamish' of interactions, how do I best query it?"

DATA COMBINATIONS

One strategy is combining diverse data sets around focused questions. For example, Ideker decided to conduct a Y2H screen that would pull out interactions involved in the mitogen-activated protein kinase (MAPK) signalling cascade — an important drug target that regulates processes such as cell growth, differentiation and survival. Ideker and his colleagues picked 150 proteins associated with the pathway and hunted for their interaction partners using Y2H assays. This revealed more than 2,000 interactions among about 1,500 proteins.

From these they selected a dozen or so proteins that had not previously been associated with the MAPK cascade and used RNA interference to knock down the expression of the identified interaction partners. In about one-third of the cases, RNA knockdown altered gene expression within the cascade, indicating that these interactions were functional. Follow-up studies provided the first experimental evidence that a protein called NHE-1 served as a MAPK scaffold¹⁰.



"It's the superposition of biophysical and functional data that is going to save the day."

Trey Ideker

Ithaca, New York, showed how combining data about protein-protein interactions and protein structure could suggest how certain mutations cause disease¹¹.

They combined several established data sets of protein-protein interactions, the physical structure of those interactions, and genetic measurements to show that when mutations do not prevent proteins from being expressed but still cause disease, they are more likely to occur in the interface between interacting proteins than elsewhere. "For the past decade, biologists have been using this mathematical definition. Every protein is a mathematical dot. But we know that protein structure is fundamentally important for function," says Yu.

Information about whether an interaction occurs in a specific cell type or under certain

conditions could go a long way to revealing its function, says Anne-Claude Gavin, who studies protein complexes at the European Molecular Biology Laboratory in Heidelberg, Germany. "Interactions have to be context-dependent; they have to start at one time and stop at another." But these studies are difficult and are rarely done. "This is a level of sophistication that we just don't understand," she says.

To understand a protein-protein interaction in context, researchers need to single them out for focused studies.

Sometimes, screening techniques can be adapted to follow particular interactions in depth. For example, complementation assays with fluorescent proteins or luciferase can be used to follow interacting proteins. Because different coloured fluorescent proteins are so similar, one protein can be tested for interactions with two or more proteins in the same cell. One protein is labelled with a fragment of yellow fluorescent protein, a second with a fragment of cyan fluorescent protein and another interrogated protein carries a fragment common to both fluorescent proteins. This can show which protein interactions are occurring and where in the cell they occur. Complementation assays with luciferase can also be used with multiple colours of proteins and have the advantage that the enzyme easily breaks apart and reforms, allowing researchers to study how interactions can be disrupted. Imaging techniques such as bioluminescent resonance energy transfer and fluorescent resonance energy transfer can be used in living cells. They use genetically tagged proteins that emit light when proteins come into contact with each other, and so are used in a variety of assays. Other assays label each of two proteins and then monitor whether they move together in cells.

Although slower and more expensive than large-scale screening efforts, one-at-a-time explorations of interactions are essential, says Uetz. "Eventually you want to drill down into the actual interactions." ■

Monya Baker is technology editor for *Nature and Nature Methods*.

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Ectopic foci in pulmonary veins can trigger episodes of atrial fibrillation (AF), but how these foci arise remains unclear. This review highlights the role of the autonomic nervous system in the induction and maintenance of atrial arrhythmias, and explores neural modulation as a treatment strategy for AF.

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L. Chen, D. J. Magliano & P. Z. Zimmet

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S. Ogino, J. Galon, C. S. Fuchs & G. Dranoff

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J. D. Greenberg, V. Furer & M. E. Farkouh

Comorbid cardiovascular disease is a substantial problem for patients with rheumatoid arthritis (RA). Here, the authors summarize the evidence—and knowledge gaps—regarding the influence of biologic therapies on cardiovascular risk parameters in patients with RA.

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Image-guided radiation therapy for muscle-invasive bladder cancer

J. Thariat *et al.*

The hollow and mobile nature of the bladder causes variations in its volume, shape and position, making delivery of radiation therapy to bladder tumors challenging. Here, Thariat *et al.* describe using imaging to improve the accuracy and effectiveness of radiation therapy for bladder cancer.

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INNOVATION

The big idea of technology transfer

Working at the interface between science and business offers an opportunity to bring ideas to market.

BY CHARLOTTE SCHUBERT

As Angela Loihl worked her way through her graduate research project at the University of Iowa in Iowa City, she noticed the scope of her studies getting narrower. "I spent all my time learning more and more about less and less," she says of her research in mice, which focused on a protein thought to have a role in stroke. "I questioned

how relevant that was to the human condition."

Fourteen years after she earned her PhD, her career is far broader. As a technology manager at the Center for Commercialization at the University of Washington in Seattle, she covers a wide range of life-science fields — from microbiology to radiology, with an occasional foray into chemical engineering.

Loihl's role is to take scientific ideas from academia and negotiate their transfer to

biotechnology start-ups and pharmaceutical companies, at which point the most promising leads may become new therapies. Loihl is routinely presented with intriguing, and sometimes thorny, questions. What is the commercial potential of a particular university technology? Which company will license the rights to which innovation? Are a researcher's findings strong enough to launch a spin-off?

Loihl has helped to foster technology related to influenza treatments, tests to assess the risk of cardiovascular disease and a vaccine for the type 2 herpes simplex virus. "Every day I learn something new from the science and business perspective," she says. "I love this job."

Working in technology transfer demands teamwork and the ability to assess a huge range of scientific areas. Those who thrive have the right mixture of extroversion, scientific breadth and business sense. They are also able to juggle multiple projects at once. The hardest thing, says Loihl, is keeping everyone's expectations realistic, such as ensuring a researcher does not overestimate the commercial value of his or her work, or that a company does not underestimate it. "If you do something well by one stakeholder, it usually means you are upsetting another," says Loihl, adding that the variety of duties and challenges is what makes her job great.

WHERE THE JOBS ARE

Fourteen years ago, there were 26 employees at what was then called the office of technology transfer at the University of Washington. Now there are 54. The growth, which is not atypical for large academic institutions in the United States, is due in large part to the Bayh-Dole Act of 1980. The act changed the pace and manner of innovation in the United States, giving universities and not-for-profit organizations control over the intellectual-property rights of federally funded research done within the institution. The result was a huge increase in technology-transfer related opportunities, particularly in the 1990s as universities mined this new source of revenue.

The expanding offices have taken on functions beyond the usual roles of patenting and licensing technology, resulting in new types of work. At the Center for Commercialization, office head and entrepreneur Linden Rhoads leads a team of 15 technology managers (also known as licensing managers or licensing specialists), which are typical positions for people making the jump from science, as Loihl did. Rhoads also employs specialists who team ▶

► up with the managers on various projects. "We have all kinds of people in support roles," she says.

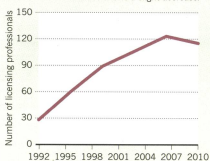
For instance, three industry-relations officers help the technology managers to understand industry needs and to forge collaborations between faculty members and companies. Most of these officers are scientists or engineers with extensive business experience. Four people, most of whom have business experience at small start-up companies, are in a 'New Ventures' group, which works with technology managers and researchers to start companies by inviting potential investors to the office, for example, and recruiting business people for an 'entrepreneurs-in-residence' programme. The New Ventures group also boasts a grant writer, who can pull in funding from sources such as the US Small Business Administration in Washington DC to foster a start-up.

Patent agents in Rhoads' team, some of whom have law degrees, write the lengthy and technical patent applications and handle the continual back-and-forth between the patent office and researchers to keep the application moving forward — a task that technology managers often perform in smaller offices. Loihl advises job seekers to "scrutinize the office to see what kind of resources are available".

Although Rhoads is still hiring staff, the big expansion in the number of US technology-transfer jobs has slowed, says Robin Rasor, director of licensing at the University of Michigan in Ann Arbor and former president of the Association of University Technology Managers in Deerfield, Illinois (see 'Growth subsides'). The association's annual survey of technology-transfer offices at US universities, hospitals and research institutions showed a slight drop in the number of

GROWTH SUBSIDES

Number of licensing professionals at eight major US research universities shows a slight decrease.



full-time licensing jobs from 1,041 to 1,018 between 2009 and 2010, at the 172 institutions that responded in both years. Job markets in Germany and the United Kingdom, which have long had laws similar to Bayh-Dole, are likewise slowing, says Anders Haugland, president of the Association of European Science and Technology Professionals, based in Leiden, the Netherlands. But ongoing changes in patent law and technology-transfer policies elsewhere have created job potential, notes Haugland, who also heads a technology-transfer office of seven institutions, including the University of Bergen and Haukeland University Hospital, in Norway.

Norway adopted policies similar to the Bayh-Dole Act in 2003. The countries universities now retain one-third of the proceeds from intellectual property arising from research conducted on their campuses. Previously, faculty members generally retained the full rights. Back then, Haugland's technology-transfer office did not even exist. It now has 23 employees, some of whom have

come from as far as India, Croatia and Lithuania. "We are hiring people every year and we are growing," says Haugland.

GETTING THE JOB

As Loihl became disillusioned with bench science, she began inquiring about internship opportunities at the then-small technology-transfer office at the University of Iowa, but they showed little interest. "They didn't know how they would use me," she says.

But times have changed. Technology-transfer offices now offer various paid and unpaid internship programmes. The University of Washington has a typical technology-transfer intern programme. Interns prepare summaries about the university researcher's innovations to facilitate licensing negotiations, and help to evaluate whether a new technology can be patented and whether there is a potential market for it. Often an internship can lead to a longer, salaried apprenticeship, says Rhoads. A technology-transfer role may also lead to a position in industry (see 'Jumping to industry').

An internship is the best way to get a foot in the door, especially for those making the transition from science, says Rasor. She and Rhoads have both hired interns who came straight from postdoctoral fellowships or graduate programmes as junior technology managers.

Courses in business, marketing or law can help to land a job or internship. PraxisUnico, a not-for-profit organization in Cambridge, UK, offers a three-day 'fundamentals of technology transfer' course, which is popular for those new to the sector, says Alison Campbell, a consultant and chair of PraxisUnico's training committee. Aspiring technology managers can learn the basics of patenting, trademarks and licensing, as well as marketing and negotiation tactics.

Although not all of Rhoads' team have a masters in business, she says that the degree can help with tasks such as assessing the market potential for a technology — for example, by researching the size of the market, cost of goods or pricing models. Rhoads herself has a law degree, which helps in understanding the complicated language of contracts. But in the end, it may be business experience that carries the most weight. "If you have someone who has any kind of experience in industry, that is a big plus," says Rasor. Experience in a business office is preferable, but even work as a researcher at biotechnology company can help, she says.

There is no typical route into a technology-transfer role. And ultimately, less-tangible abilities, such as communication and people skills, could be what leads to success in the field. "People need to be good at having consultations with scientists, turning over rocks and introducing researchers from industry to academic researchers," says Rhoads. Those who thrive in the job, she says, are "natural connectors". ■

Charlotte Schubert is a freelance journalist based in Seattle, Washington.

JUMPING TO INDUSTRY

Making the move to the private sector

Working in a technology-transfer office does not mean staying in academia. Some use their technology-transfer experience to make the leap to the private sector.

"We are at the other side of the deal," says Polly Murphy, a vice-president at drug firm Pfizer in San Diego, California, who leads 30 people in the business development team for the company's research and development branch. The team negotiates transactions with other companies, not-for-profit organizations, universities and other institutions. Most of the team came from the technology-transfer world, which, says Murphy, is the best place to learn how to draft licensing and collaboration agreements. Working in technology transfer can also lead to a job in

a contracts and outsourcing office, which manages agreements for clinical trials and other projects. But Murphy cautions that shrinkage in the industry has made such jobs scarce. "In my department nobody ever leaves," she says.

Other technology-transfer employees head to biotechnology firms or use their scientific knowledge and understanding of the market to work in a venture-capital firm. But biotechnology start-ups come and go, along with their licensing offices. Those who are risk averse should be wary of the jump to industry, says Anders Haugland, head of the Association of European Science and Technology Professionals in Leiden, the Netherlands. And universities and research institutions often have better benefits. **C.S.**

COLUMN

Rating research risk

Too many young physicists embark on projects without knowing the risks. There is a better way, argues **Abraham Loeb**.

In physics, the value of a theory is measured by how well it agrees with experimental data. But how should the physics community gauge the value of an emerging theory that cannot yet be tested experimentally? With no reality check, a less than rigorous hypothesis such as string theory may linger on, even though physicists have been unable to work out its actual value in describing nature.

This sort of uncertainty has implications not only for the gathering of knowledge for the scientific enterprise, but also for fledgling physicists. The investment of research time in strong intellectual assets is crucial for graduate students who want to establish their careers on a good foundation. But not all young researchers are aware of the history that accompanies every research area. They often have to rely on word of mouth from their PhD adviser or colleagues for this information.

What if the physicists could call on a ratings agency, not unlike a lender would do before deciding whether to offer credit? I am advocating the creation of a website that is operated by graduate students and that will use various measures of publicly available data (such as the number of newly funded experiments, research grants, publications and faculty jobs) to gauge the future returns of various research frontiers.

THEORY BUBBLES

The study of the cosmic microwave background provides an example of how theory and data can generate opportunities for young scientists. As soon as NASA's Cosmic Background Explorer satellite reported conclusive evidence for the cosmic microwave background temperature fluctuations across the sky in 1992, the subsequent experimental work generated many opportunities for young theorists and observers who joined this field. By contrast, a hypothesis such as string theory, which attempts to unify quantum mechanics with Albert Einstein's general theory of relativity, has so far not been tested critically by experimental data, even over a time span equivalent to a physicist's career.

Senior scientists might seem the people best suited to rate the promise of research frontiers. But too many of these physicists are already invested in evaluating the promise of these speculative theories, implying that they could have a conflict of interest or be wishful thinkers. Having these senior scientists rate future promise would be akin to the 'AAA' rating that financial



agencies gave to the very debt securities from which they benefited. This unseemly situation contributed to the last recession, and a long-lived bias of this type in the physics world could lead to similarly devastating consequences — such as an extended period of intellectual stagnation and a community of talented physicists investing time in research ventures unlikely to elucidate our understanding of nature — a theory 'bubble', to borrow from the financial world.

Of course, graduate students are busy. But they could serve a limited term of service for maintaining the site and be government-funded. For example, students supported by US National Science Foundation fellowships maintain astrobits.com, a website that summarizes new astrophysics papers.

CREDIT RATING

The physics 'credit-rating' website would use evaluation metrics to factor in, with the correct weighting, all the ingredients that would ultimately make scientific research successful.

For physics, this might include the existence of an underlying self-contained theory from first principles, the potential for experimental tests of this theory and a track record of related research programmes. Clearly, factors such as intellectual excitement cannot be quantified, but as long as funding agencies are supporting projects and the information provided is accurate, the data about the growth of a field should echo this 'excitement' factor.

The evaluation metric would have to be predetermined and supported by numbers that are based on archival data gathered through automated searches for keywords in electronic data

archives (see arxiv.org or nsf.org). Aside from automated searches, practitioners from fields that are being evaluated could submit supplementary data that would be incorporated into the analysis.

The entire data set would include the level of funding allocated to experiments and research grants, the status of the underlying theory and the number of publications and faculty jobs within the particular field of research. The simplest model relates the change in these parameters to a linear combination of their values. For example, the publication rate is expected to relate to a linear combination of the number of experiments, faculty jobs and available research funds. With the right mix of time spent on theory, experimental work and grant support, a research frontier would show exponential growth in this linear model. The next step would be to calibrate this model using historical data about the growth of successful research frontiers.

The website could be helpful to institutions and governments, not just to individual scientists. A balanced assessment of the level of risk and potential benefits from emerging research frontiers can increase the efficiency of the workforce, leading to stronger growth. And it could help funding agencies to optimize their allocation of money to promote progress in research. In fact, it would be in the interests of funding agencies to support the website and help the students to take part (for example, through special grants or fellowships).

The website might also convince senior researchers to shift their focus to new research areas, perhaps as a result of the influence that the rating procedure may have on funding agencies. But maintaining balance and ensuring diversity among subfields, taking some risks and avoiding funneling resources into a small number of successful but conservative programmes are important considerations for funding agencies (A. Loeb *Nature* **467**, 358; 2010).

Nearly every worthwhile endeavour involves some risk. But mitigating that risk, and helping young scientists to make informed decisions about the field in which they should invest their time and intellect, would yield a more efficient scientific enterprise. ■

Abraham Loeb is chair of the astronomy department and director of the Institute for Theory and Computation at Harvard University in Cambridge, Massachusetts.

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The Channing Division of Network Medicine at Brigham and Women's Hospital seeks to recruit 2-3 new faculty in the areas of systems biology and network medicine to help structure this new program by working with existing faculty who have expertise in statistics, bioinformatics, epidemiology, genetic epidemiology, and genomics of complex human diseases. Channing is the home of the ongoing Nurses' Health Study, with 35 years of follow-up and repeated detailed information on diet and lifestyle from 120,000 women. From this and many other cohorts, we have DNA samples from more than 300,000 individuals. The Division has its own freezer storage system and extensive computer resources.

Professor or Associate Professor (1 Appointment)

The Division seeks applicants at the professor level to help structure this new program by working with existing faculty who have expertise in statistics, bioinformatics, epidemiology, genetic epidemiology, and genomics of complex human diseases. Expertise in computational biology is preferred. The successful applicant will conduct his/her own research and collaborate with over 50 current faculty and almost 200 support staff. The goal is to develop a program in network medicine that will collaborate with multiple divisions within the Department of Medicine at BWH.

Instructor/Assistant Professor (1-2 Appointments)

The Division seeks junior faculty applicants looking to build their own research programs that will, at least in part, leverage the extensive study populations, biological samples, and genetic/genomic data which currently exist at the Division. They will develop collaborations with existing faculty who have expertise in statistics, bioinformatics, epidemiology, genetic epidemiology, and genomics of complex human diseases. Expertise in computational biology is preferred. Appointment at Harvard Medical School is expected at an academic rank commensurate with the applicant's experience.

Applicants should send a letter detailing their interest and CV to the Chair Search Committee by June 1, 2012.

Scott T. Weiss, M.D., M.S.

Professor of Medicine

Associate Director, Channing Laboratory

Director, Partners Center for Personalized Genetic Medicine

181 Longwood Avenue, Boston, MA 02115

c/o Mellie Sanchez

Email: mellie.sanchez@channing.harvard.edu

Brigham and Women's Hospital and Harvard Medical School are equal opportunity employers; therefore, women and minority applicants are especially encouraged to apply.



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- ES Cell Biology Technician

If you are interested and would like to be considered for the post, please apply to careers@kymab.com with a covering letter and your CV outlining your suitability for this role, along with the corresponding reference number.

U223591R

nature chemistry

Associate Editor

Nature Chemistry is a journal dedicated to publishing high-quality original research papers in all areas of chemistry. In addition, *Nature Chemistry* also features commentary and analysis from and for the wider chemistry community. For more information about the journal, see our website (<http://www.nature.com/nchem>).

We are looking for an associate editor to join the editorial team. The ideal candidate will have a PhD in a chemistry-related discipline. Postdoctoral experience will be an advantage but is not required; emphasis will be placed on broadly trained applicants with a good knowledge of the chemistry community.

Key elements of the job include the selection of manuscripts for publication, management of the peer-review process, as well as commissioning and editing content for the journal (review articles, News & Views articles, features and commentaries). Associate editors are also expected to write editorials, research highlights and book posts. Close contact with related research communities, through conferences and laboratory visits, will be an essential component of the job.

This is a demanding and intellectually stimulating role that calls for a highly motivated individual with a broad interest in science and publishing.

The position will be based in our London office.

Applicants should visit <http://jobs.macmillan.com/> and upload the following to vacancy reference NP3/029/12 – a CV, a research highlight in *Nature Chemistry* style (approximately 250 words) on a recent relevant paper in the literature; and a cover letter explaining their interest in the post and their salary expectations. Please do not send thesis chapters or research papers.

Closing Date: Friday 20th April 2012

nature publishing group **npg**

IN201130R

Director, UCL School of Pharmacy

The School of Pharmacy merged with UCL in January 2012 to form an academic division within the Faculty of Life Sciences known as the UCL School of Pharmacy. The School of Pharmacy was founded 170 years ago and remains dedicated to teaching and research in pharmacy and pharmaceutical sciences. It is now at the heart of Europe's largest grouping of biomedical research. The School employs over 200 staff, and has a current annual turnover of over £20m.

UCL invites applications for the post of Director from candidates of international standing, with an outstanding track record in leading interdisciplinary research and substantial experience of academic and strategic management. The person appointed will be a charismatic leader with clear vision, a strong strategist, and a first class manager of people and complex relationships.

This full-time appointment is available from September 2012. The post of Director carries with it a professional position within UCL. The salary will be according to experience and seniority on the non-clinical professorial scales.

Enquiries may be made to Professor Mary Collins (Dean, Faculty of Life Sciences), email: mary.collins@ucl.ac.uk; tel: 020 7679 0883; or Dr Brian Pearce (Acting Director, UCL School of Pharmacy), email: b.pearce@ucl.ac.uk; tel: 020 7753 5819.

For further details about the vacancy and how to apply online please go to <http://www.ucl.ac.uk/hr/jobs/> and search on Reference Number 1241853.

Closing Date: Friday 4th May 2012.

Interview Date: by mutual convenience in June 2012.

We particularly welcome female applicants and those from an ethnic minority, as they are under-represented within University College London at this level.

U223430R

Academic position in Genetics and Molecular Biology of Animals Department of Life Science – Faculty of Sciences GIGA-R University of Liège

The University of Liège (Belgium) invites applications for an academic position in genetics and molecular biology of animals in the department of Life Sciences, Faculty of Sciences (www.facsc.ulg.ac.be) and GIGA center (<http://www.giga.ulg.ac.be>). Applicants must possess a PhD, a distinguished record of scientific research and extramural funding, administrative and leadership skills, and a strong commitment to education and academic excellence. Applicants should be able to teach in English but are also expected to teach in French, possibly after a transition period. The candidate will work in GIGA-R and will be expected to foster new areas of research while supporting and enhancing current research strengths.

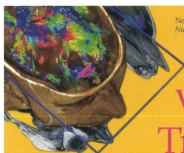
Nominations and inquiries may be directed to Mme F. MOTTE, Faculty's Administrative Manager - tel. : +32 4-366 36 52 - Francoise.Motte@ulg.ac.be. Candidates should submit, by registered letter, their application to Professor Bernard Rentier, Rector, University of Liège, Place du 20 Août 7, B4000 Liège, Belgium. The application should be sent before May 31st 2012 and must include a letter of interest, a curriculum vitae, a complete list of publications and a research project of five to ten pages.

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Neural tracts in the brain.
Nasdaq Medical/Wellcome Images

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U223502R

nature neuroscience

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Director, Centre for Psychiatry and Neuroscience (CPN) in Sainte-Anne Hospital, Paris, France

Inserm, University Paris Descartes (recently identified and funded as one of the 8 French Universities of excellence) and Sainte-Anne hospital invite applications for the position of Director of CPN.

The CPN provides an excellent scientific environment that allows neuroscientists and clinicians to work in close proximity to each other, facilitating the path from bench to bedside. It is located in Sainte-Anne hospital which has a long record of excellence in psychiatry and clinical neurosciences and attracts clinical and basic science students and visiting scholars from around the world. Sainte-Anne hospital drains the largest clinical population for psychiatry in France, and for cerebrovascular diseases in the Paris area. The CPN is currently home to 80 scientists and investigators, 30 technical support staff and 40 trainees, spanning the spectrum from basic to clinical research, particularly in translational and biological psychiatry, cerebrovascular sciences, and cognitive neuroscience. Laboratory-based research programmes are currently located in a 2,800 Sq-meter building, with in-house molecular and cellular biology, imaging, and behavioural facilities and animal house. Laboratories are planned to move into a new building of 5,000 Sq Meters, scheduled for occupancy in 2014. Clinical research facilities (including biobank, clinical research centre, human brain imaging (1.5 and 3T MR)) are also available.

The Director will be an eminent scientist, MD, PhD or both, with a track record of excellence in fields relevant to psychiatry and/or translational neurosciences, and documented leadership credentials in an academic research setting. He/She will serve as Chief Scientific and Executive Officer, with a mandate to expand the Centre's existing strengths, and enhance the international stature of the CPN. He/She will be expected in parallel to carry out his/her own research in the Centre, whose facilities will be made available.

Applicants should send an expression of interest, research statement (not exceeding 5 pages), curriculum vitae and three letters of recommendation or names of references to maurice.nakache@inserm.fr, at Centre for Psychiatry & Neuroscience, 2 ter rue d'Alésia 75014, Paris, France. To receive full consideration, applications should be submitted by June 3rd, 2012. Salary and position will be tailored to the qualifications of the successful applicant.

W223527R

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Energy & Environment Hire

Tenure-track position in the field of Energy and Environment at **assistant, associate, or full professor** level.

As part of Duke University's new Energy Initiative, the Division of Earth and Ocean Sciences in the Nicholas School of the Environment invites applicants for a tenure-track faculty position at the assistant, associate or full professor level in the field of Energy & Environment. The candidate should have a strong science or engineering background, an understanding of energy issues beyond his/her area of technical training, and the ability and interest to interact with colleagues from a wide spectrum of fields on interdisciplinary energy problems. We are particularly interested in candidates who have expertise in energy systems, i.e. the integrative use of energy by humans, including energy resources, power generation and distribution, energy technologies, and the connection between energy use and climate change. The candidate's work will ideally reflect a long temporal (decadal and greater) and large spatial (regional to global) scale perspective on energy systems.

If a senior candidate is hired, he/she will assume Directorship of the Nicholas School's Energy & Environment Program, which encompasses leadership of the Energy & Environment Concentration in the School's Professional Masters of Environmental Management Program, and leadership roles in Duke University's Undergraduate Certificate in Energy & Environment and in the University's Gendell Center for Engineering, Energy & Environment, the latter two of which are jointly administered by the Nicholas School and Duke's Pratt School of Engineering. The successful candidate will also join the Faculty Advisory Council of Duke's new Energy Initiative. This Council consists of energy experts from across the University and is responsible for developing, coordinating, and promoting a shared set of energy courses and programs, and energy faculty hiring at Duke. More on the Initiative can be found at energy.duke.edu.

The Nicholas School focuses on leadership in education, research, and service to understand basic earth and environmental processes, to understand human behavior related to the environment, and to inform society about the conservation and management of the environment and its natural resources. Research interests within Earth and Ocean Sciences and the Nicholas School that will complement this position include climate dynamics, water resources, land-use change, and environmental science and policy. Additional interactions with respect to energy are possible with the Pratt School of Engineering, Trinity College of Arts & Sciences, the Fuqua School of Business, the Sanford School of Public Policy, the Law School, and the Nicholas Institute of Environmental Policy Solutions.

Letters of interest should include a curriculum vita, a statement describing the candidate's research interests and goals as they relate to energy systems, and names of three references. All materials should be sent electronically as a single PDF file to Mrs. Mary Anne Perez at maryanne@duke.edu.

The search will remain open until the position is filled, with a target start date of September 1, 2012.

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Brigham and Women's Hospital Neuropathology Division



Brigham and Women's Hospital



Harvard Medical School

NOTICE OF RESEARCH FACULTY POSITION

Rebecca D. Folkert, MD
Director
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75 Francis Street
Boston, MA 02115
Tel 617-732-7532
Fax 617-975-4944
rfolkert@partners.org

The Division of Neuropathology in the Department of Pathology at the Brigham and Women's Hospital, Harvard Medical School and the Dana-Farber Cancer Institute are seeking a full-time Academic Neuropathologist to be hired at the level of Assistant Professor or higher. The ideal applicant is expected to have a strong track record of accomplishment as a physician-scientist, be board certified in Neuropathology and Anatomic Pathology, and have an M.D./Ph.D. or M.D. degree. The candidate will be expected to establish an independent laboratory research program focused on human cancer biology, including neuro-oncology. Expected start date would be summer of 2012. The position will combine clinical sign-out duties in adult and pediatric surgical and autopsy neuropathology at the Brigham and Women's Hospital and Children's Hospital Boston. Candidates would be expected to take advantage of the extensive resources and collaborative interactions within the Brain Tumor Research and the Neuroscience Programs of the Harvard Medical School, Dana-Farber Cancer Institute, Brigham and Women's Hospital, and The Children's Hospital Boston.

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Ivana Delalle,
MD, PhD

Mel B. Feany,
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MD, PhD

Sandro Santagata,
MD, PhD

Applicants should submit a full Curriculum Vitae and cover letter via email to: Dr. Keith L. Ligon MD, PhD, Vice-Director of Neuropathology, Brigham and Women's Hospital, kligon@rics.bwh.harvard.edu

Brigham and Women's Hospital is an equal opportunity/affirmative action employer. Review of applications will begin on April 1, 2012 and will continue until the position is filled.

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For more information and a full vacancy text:

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NATURE ISSUE DATE:

26 JULY 2012

DEADLINE FOR ADVERTISERS:

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WITS 21st Century Institutes Programme

GLOBAL CHANGE AND SUSTAINABILITY RESEARCH INSTITUTE.

1. EXXARO CHAIR and DIRECTOR

The University of the Witwatersrand invites applications for the post of Director of the newly established Global Change and Sustainability Research Institute (GCSRI). The candidate appointed to the Directorship will also hold a Chair in his or her field of specialisation.

The GCSRI is one of six major new transdisciplinary Research Institutes being set up by the University as a strategic contribution to the challenges faced by South African and African societies in the 21st Century. The GCSRI already houses a number of research projects. Owing to the generosity of Exxaro, we are pleased to be able to announce the consolidation of the Institute through the appointment of a Director and Chair.

The establishment of the GCSRI is a significant marker of the University's intention to lend prestige and standing to the area of Global Change and Sustainability Research.

REQUIREMENTS: The appointed Director and Chair will have:

- an international reputation, based largely on an outstanding record as a researcher
- sufficient academic and/or professional standing to command the respect and confidence of the members of the Institute and University.
- experience at a senior management level
- academic vision and leadership,
- experience in the management of research
- a good track record of fund-raising for large projects

He or she will be expected to:

- build the Institute into a substantive centre of international standing through significant fund-raising, in cooperation with the 21st Century Institutes programme as a whole
- consolidate and manage the existing programmes within the Institute
- build partnerships both locally and internationally
- develop an original and challenging research programme, taking into account the requirements of donors

Thus the Directorship will involve the overall management, consolidation and expansion of the Institute, co-ordinating its postgraduate teaching, research and administrative activities; planning and promotion of academic activities in the wider community; human resources recruitment and management; and financial and infrastructure resource management.

The University wishes to make a five year appointment in the first instance. It is anticipated that an extended appointment would be on offer thereafter, subject to the successful performance of the Director and the continuing availability of funds. The Director will report to an appropriate Deputy Vice-Chancellor and to an Advisory Board.

QUALIFICATIONS: An appropriate PhD, a significant publication record; relevant administrative and research management experience; fund-raising experience; experience within a university academic environment will be considered favourably.

SALARY & BENEFITS: An attractive and competitive salary package will be negotiated on an individual basis. In the event a candidate from outside of South Africa is appointed, relocation and settling-in allowances are payable. Any prospective appointee who is not a South African citizen will be required to obtain a work permit before taking up the post.

FOR FURTHER INFORMATION: Please contact Professor AM Crouch on +27 11 717 6012 (GMT + 1 hour) or Andrew.Crouch@wits.ac.za

TO APPLY: Please submit curriculum vitae, including publications, the names and electronic contact details of four referees and a motivation not exceeding two pages to Ms Noxolo Mavunda at recruiting.centralHR@wits.ac.za

THE CLOSING DATE for applications will be the 4th May 2012.



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NOR CUSTOM STALE

An antic disposition.

BY ANATOLY BELILOVSKY

Old people move slowly. A knee may work just fine today and buckle tomorrow. A tiny turn can change a functioning joint into a monolithic block of agony.

Old people need to remember things like that.

"You look great," said Bob, looking down and chewing at his dentures.

Bob had been a good striker, back in the day, but he never learned to fool goalies. He always looked where he wanted the ball to go. So in the end, I went pro, and Bob went into insurance.

"Really good," Bob added. His feelings littered his face the way his lunch decorated his tie: a forced tight, guilty smile with pity in the tilt of his head, and a tiny trace of *Schadenfreude* in the crow's feet around his eyes.

I knew how I looked. I looked terrible: slumped shoulders, shuffling gait, shabby jacket, stained pants.

"Thanks," I whispered. "Look at us. Sixtieth reunion. Who'da think we'd live this long, eh, roomie?"

Bob looked up, his smile genuine now. "Athletes don't age as fast," he said. "I still play nine holes, every week. Like clockwork. Keeps me young."

I nodded. "It's working," I whispered. "You haven't changed a bit."

He barked out a single laugh. "Right. Not a bit," he said. Leaning back, he turned his head slowly through a short arc, sweeping his gaze over the far side of the quad. "Princeton sure has changed," he continued. "There's dorms where soccer fields used to be. Where you and I played. Mixed dorms! Not just co-ed. Mixed. Ain't that something!" He slapped his knee, winced slightly.

I nodded again.

"Trouble with your voice?" he asked.

"Sort of," I whispered.

He sighed. "Joanie had a stroke, she can't talk at all," he said. "And Todd died last year of throat cancer, he had a tracheostomy. Had to plug the hole in his neck when he wanted to say anything."

I sighed, too, and lowered my eyes. Joanie and I lived together, sophomore year. I'd never fool her for a minute.

"I worked as long as I could, only retired after I got my bypass," he said. His face took on that look again: guilt, pity and a dash of gloat. Same look he had when I'd told him

Joanie had left me. Minus the dentures and the hand tremor. "Joanie gets round-the-clock nursing. Government pays for everything," he added. "Wouldn't wanna get old in a country where you pay for medicine."

Joanie moved in with him a week later. Maybe that was why I went overseas, so I'd never have to see that look again. In either of their faces.



Bob reached for my shoulder, his fingers trembling as if rolling an invisible cigarette. I fought the urge to move away.

"I heard health care is expensive where you live," he said.

I nodded, with far more force than I expected.

Bob looked up sharply.

I winced and rubbed my neck a fraction of a second later. Bob leaned closer to me. His shiny dentures clashed with his cracked, spit-flecked lips; his eyes, once brown, were now ochre iris on yellow sclera.

"You could get your citizenship back," he said, barely moving his lips. "My grandson is a damn good immigration lawyer."

I shrugged.

"Think about it," he said.

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"I will," I said. "But now I have to go. Don't want to miss my flight,"
"Stay with me," he said quickly. "We've

guest rooms at the home. We can go see Joanie tomorrow."

I counted to five and held my breath: an old voice actor's trick.

"No," I said.

The word came out as I intended: with longing. With reluctance. With regret.

Bob shook my hand and hobbled away as quickly as he could.

I had to sprint across the terminal to reach my gate on time. People stared. My wrinkles itched; I detoured to the lavatory to peel them off. The TranSec agent looked at me with a suspicious squint.

I called the Farm from the plane while it waited for clearance. Gulnara answered.

"Well, hello, stranger," she purred. "Do we have a date?"

"Sure," I said. "When can you fit me in?"

"You want a quickie," she said, "or the whole jalapeño?"

"Enchilada," I said. Gulnara's English was perfect. It was American she had trouble with. "I want the whole enchilada. It's been a while."

"So I see." She paused; I heard keys tapping. "I have an opening Wednesday. Is this good?"

"Sure," I said. "Training camp doesn't start for another fortnight."

I heard tapping again. "Excellent. Full rejuvenation, a five-day course starting Wednesday, shall I debit your fee now?"

"Go ahead," I said. "Did it go through?"

"With your credit rating?" she said. "Of course it went through." She paused. "I'm so glad you haven't retired. Watching you play — it never gets old. It's like, you are not just playing soccer, but also poker and chess at the same time. Does this make sense?"

"Sweetheart," I said, "I can't afford to retire."

Her answer drowned in the turbine spin-up. I disconnected my phone, leaned back, turned on the viewscreen. The plane made a climbing turn above central Jersey before heading over the Atlantic. Somewhere below, shabby, weed-choked Princeton sweltered in the heat, and Bob shambled with a cane to the train that would take him to his nursing home.

Pity about poor Bob. ■

Anatoly Belilovsky learned English from Star Trek reruns and is now a paediatrician in New York.

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NORMAL AGING?

OR PATHOLOGICAL NEURODEGENERATION?

Using a massive brain PET database, Hamamatsu Photonics distinguishes Alzheimer's disease from normal aging.

Dementia is on the rise around the globe. Doctors hoping to maximize the effectiveness of treatments with early diagnosis and researchers scrambling to design drugs that address the causes or the symptoms are facing the same problem: How do you differentiate the normal aging processes from pathological conditions such as Alzheimer's disease?

Hamamatsu Photonics has pushed the quest for an objective, accurate diagnostic tool close to realization. Equipped with a unique database of over 6000 PET scans from normal individuals of various ages and both sexes, Hamamatsu Photonics' scientists have pieced together a detailed understanding of how the brain works and changes with time.

Based in that knowledge, the company carried out a study* comparing the brain activity of normal subjects and that of Alzheimer's disease patients using a Hamamatsu Photonics PET camera. The device picks up subtle differences in brain activity at distinct regions throughout the brain.

Hamamatsu Photonics converted that data into an easy scoring method that can discern quickly and simply between the groups and with unprecedented accuracy. The computer-aided diagnosis (CAD) was even able to predict which patients with mild cognitive impairment would progress to Alzheimer's disease, further expanding the treatment window.

As an objective and easy-to-use tool for CAD, it will suit both doctors and researchers. However, the main beneficiary will, of course, be patients and their families.

Main visual: PET brain scan images (color) overlay MRI images (gray) to provide a comprehensive view of the brain's health. The images on the left show brain changes associated with normal aging. The images on the right show the onset of dementia, one form of which is Alzheimer's disease. Orange-to-yellow coloring indicates regions with reduced glucose metabolism.

*Kakimoto, A, et al. *PLoS ONE* 6(9): e25033. doi:10.1371/journal.pone.0025033 [2011]

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